University of Alberta

Studies on the *in vitro* Activity of Lovastatin Nonaketide Synthase

by

©

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment
of the requirements for the degree of Doctor of Philosophy

Department of Chemistry

Edmonton, Alberta

Fall 2006

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395, rue Wellington
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Your file Votre référence
ISBN: 978-0-494-22996-5
Our file Notre référence
ISBN: 978-0-494-22996-5

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Abstract

The *in vitro* activity of the lovastatin nonaketide synthase (LNKS, LovB) was examined by incorporation studies with 3-[(1*S*,2*S*,4a*R*,6*R*,8a*S*)-2,6-dimethyl-1,2,4a,5,6,7,8,8a-octahydronaphthalen-1-yl]thiopropanoic acid *S*-(N-acetylcysteamine) ester (**7**), ^{13}C-labeled sorbic acid, *S*-(N-acetylcysteamine) thioester (**17**) and 3-[(1*S*,2*S*,4a*R*,6*R*,8a*S*)-2,6-dimethyl-1,2,4a,5,6,7,8,8a-octahydronaphthalen-1-yl]thiopropanoic acid *S*-coenzymeA ester (**12**). *In vitro* attempts to incorporate these precursors, by *Aspergillus nidulans* containing *lovB* or *lovB+C* genes, into dihydromonacolin L (**1**) were unsuccessful as the compounds were either hydrolyzed or recovered unchanged from the reactions.

The pyrones 4-hydroxy-6-(1-methylheptyl)-2*H*-pyran-2-one (**18**), 4-hydroxy-6-(3-methylnonyl)-2*H*-pyran-2-one (**19**) and the unsaturated version 4-hydroxy-6-[(1*E*,3*E*,5*E*)-1-methylhepta-1,3,5-trien-1-yl]-2*H*-pyran-2-one (**3**) were successfully synthesized as standards. *In vitro* incorporation of the substrate ^{14}C malonyl-CoA, by LovB or LovB+C, into **18**, **19** or **3** was not achieved. *In vitro* attempts to incorporate the ^{13}C-labeled triketide **17** into the labeled pyrones **18a** and **19a** were unsuccessful. The O-methylated analogs (**18b**, **32**, **67** and **37**) of each of the saturated and unsaturated pyrones were also synthesized as standards.

The synthesis of the deuterated and unlabeled *S*-[2-(acetylamino)ethyl] (2*E*,6*R*,8*E*,10*E*)-6-methyldodeca-2,8,10-trienethioate (**78** and **74**) was accomplished in order to study the mechanism of the enzymatic Diels-Alder reaction of LovB. Thermal closure of an equal mixture of the triene NAC thioesters **74** and **78** reveal an inverse secondary kinetic isotope effect of 0.93, indicating that the cyclization is concerted.

During the mechanistic investigation of the Diels-Alder reaction by GC-MS it was discovered that a small amount (2%) of the *trans*-fused *endo* ring closed product, ethyl (1*S*,2*S*,4a*R*,6*R*,8a*S*)-2,6-dimethyl-1,2,4a,5,6,7,8,8a-octahydronaphthalene-1-carboxylate (**79**), which has the same stereochemistry in the decalin ring as lovastatin (**2**), is produced along with the expected products, ethyl (1*R*,2*S*,4a*R*,6*R*,8a*R*)-2,6-dimethyl-1,2,4a,5,6,7,8,8a-octahydronaphthalene-1-carboxylate (**113**) and ethyl (1*R*,2*R*,4a*S*,6*R*,8a*R*)-2,6-dimethyl-1,2,4a,5,6,7,8,8a-octahydronaphthalene-1-carboxylate (**114**). The independent synthesis and analysis of **79** confirmed this result.

Acknowledgements

To my supervisor, Professor John C. Vederas, I would like to thank you very much for your guidance, encouragement and enthusiasm not only in science, but also in life.

I would like to thank my parents Kenneth and Linda, my brother David, the rest of my family and my friends. Without their constant support, patience and love, I could not have arrived at this point in life.

Finally, I would like to thank the present and past members of our research group for their insight and help during this project. In particular, I would like to thank Jamie Côté, Brandy Chen, Hiroyuki Morita, Belén Mayo-Martín and Kris Rathwell for their synthesis of various compounds. I am especially grateful to Steven Cobb and Viji Moorthie for proof reading this manuscript. The staff in spectral and analytical services (Department of Chemistry) are also gratefully acknowledged for their technical expertise and assistance.

This thesis is dedicated to all of the brave fungi who have sacrificed their lives for the advancement of science.

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LIST OF ABBREVIATIONS

$[\alpha]_D^{26}$	specific rotation
Ac	acetyl
Ac_2O	acetic anhydride
AcOH	acetic acid
ACP	acyl carrier protein
AIBN	α,α`-azobis(isobutyronitrile)
AMM	*aspergillus* minimal media
Anal.	analysis
APT	attached proton test
aq.	aqueous
AT	acyl transferase
Bn	benzyl
bp	boiling point
br	broad
n-BuLi	*n*-butyl lithium
c	concentration
Calcd	calculated
CAN	ceric ammonium nitrate
CD	circular dichroism
CFE	cell free extract
CoA	coenzyme A

COSY	correlation spectroscopy
δ	chemical shift in parts per million downfield from tetramethylsilane
d	doublet
Da	Dalton
DIBAL-H	diisobutyl aluminum hydride
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
DCC	1,3-dicyclohexylcarbodiimide
DCME	dichloromethyl methyl ether
DEBS	6-deoxyerythronolide B synthase
DH	dehydratase
DMAP	4-(dimethylamino)pyridine
DMF	dimethylformamide
DMP	Dess-Martin periodinane
DMSO	dimethylsulfoxide
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EI	electron impact
eq	equivalent
ER	enoyl reductase
ES	electrospray
Et	ethyl
FAD	flavin adenine dinucleotide
FAS	fatty acid synthase

GC	gas chromatography
HMBC	heteronuclear multiple bond correlation
HMDS	hexamethyldisilazane
HMG-CoA	(3*S*)-hydroxy-3-methylglutaryl-coenzyme A
HMPA	hexamethylphosphoramide
HMPT	hexamethylphosphorous triamide
HMQC	heteronuclear multiple quantum coherence
HPLC	high performance liquid chromatography
HRMS	high-resolution mass spectrum
h	hours
IPA	isopropyl alcohol
IR	infrared spectroscopy
J	coupling constant
KR	ketoreductase
KS	ketosynthase
LDKS	lovastatin diketide synthase
LiHMDS	lithium hexamethyldisilazane
lit.	literature reference
LNKS	lovastatin nonaketide synthase
m	multiplet
m/z	mass to charge ratio
Me	methyl
MeT	methyl transferase

min	minute(s)
MOM	methoxymethyl
mp	melting point
MS	mass spectrometry
MW	molecular weight
MWCO	molecular weight cut off
NAC	N-acetylcysteamine
NADPH	β-nicotinamide adenine dinucleotide phosphate (reduced form)
NaHMDS	sodium hexamethyldisilazane
NaOH	sodium hydroxide
NaOMe	sodium methoxide
Na_2SO_4	sodium sulfate
NBS	*N*-bromosuccinimide
n-BuOH	*n*-butanol
NIS	*N*-iodosuccinimide
NMO	4-methylmorpholine N-oxide
NMR	nuclear magnetic resonance
nm	nanometers
NRPS	non-ribosomal peptide synthetase
PABA	*p*-aminobenzoic acid
PDA	potato dextrose agar
PDC	pyridinium dichromate
P_i	phosphate

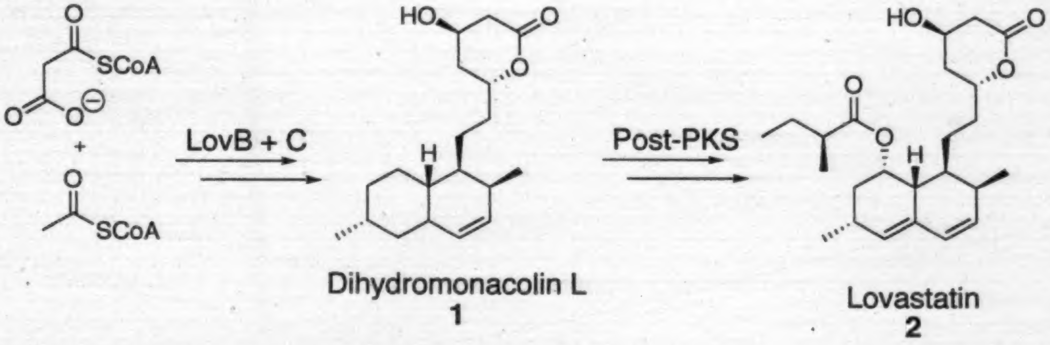
PKS	polyketide synthase
ppm	parts per million
pyr	pyridine
q	quartet
quant.	quantitative yield
R_f	retention factor
RP	reverse phase
rt	room temperature
s	singlet
SAM	*S*-adenosyl-*L*-methionine
SDS	sodium dodecyl sulfate
t	triplet
TBAF	tetrabutylammonium fluoride
TBDMS	*tert*-butyldimethylsilyl
TBDPS	*tert*-butyldiphenylsilyl
TE	thioesterase
TFA	trifluoroacetic acid
TFAA	trifluoroacetic anhydride
THF	tetrahydrofuran
THP	tetrahydropyran
TLC	thin layer chromatography
TPAP	tetrapropylammonium perruthenate
t_R	retention time

Tris	tris-(hydroxymethyl)aminomethane
Ts	*p*-toluenesulfonyl
p-TsOH	*p*-toluenesulfonic acid
UV	ultraviolet spectroscopy

CHAPTER 1. Introduction

The goal of this thesis work is to investigate the biosynthesis of the polyketide dihydromonacolin L (**1**) and thereby formation of lovastatin (**2**), a cholesterol-lowering agent produced by the fungus *Aspergillus terreus* (Figure 1).

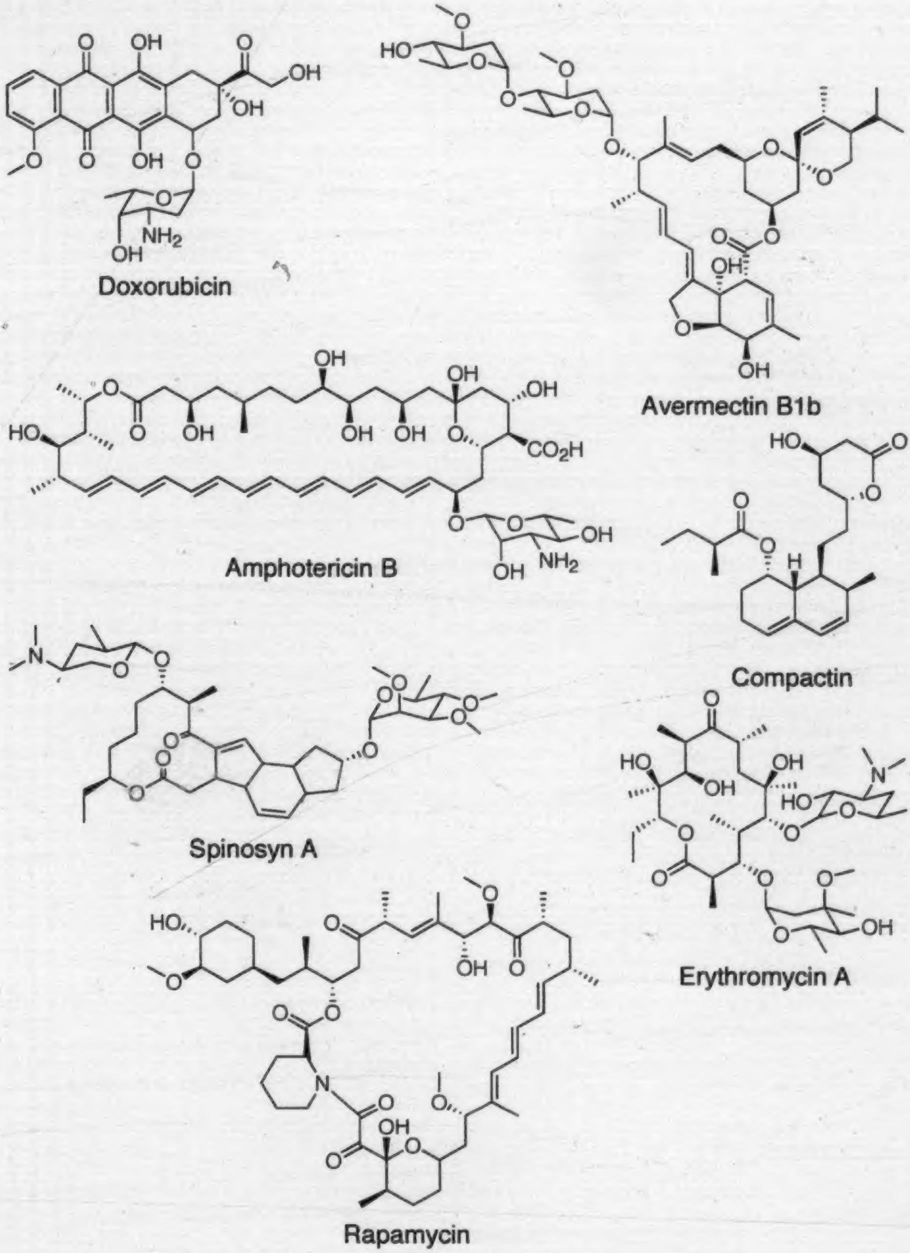
Figure 1: Lovastatin (**2**) origins



1. Polyketides and Their Biosynthesis

Polyketides[1-6] are natural metabolites originating from a wide range of organisms including bacteria, fungi and plants. These complex products stem from short chain carboxylic acids (*e.g.* acetic, propionic, butanoic and 3-methylbutanoic acids)[6] that are assembled in a head to tail fashion by polyketide synthases (PKSs), which resemble enzymes of fatty acid biosynthesis. The main difference between the two enzymatic systems is the ability of PKSs to selectively suppress reductions and dehydrations on the growing polyketide chain, whereas the fatty acid synthases (FASs) typically fully reduce the β-carbonyl to make long saturated side chains. The polyketide products that are assembled are often of medicinal/commercial importance to humans. These include antibiotics (erythromycin A[7] and tetracycline[8]), antitumour agents (doxorubicin[9]), immunosuppressants (rapamycin[10] and FK506[11]), antifungal agents (amphotericin B[12]), antiparasitic agents (avermectin B1b[13]), insecticidal agents (spinosyn A[14]) and statins (lovastatin (**2**)[15] and compactin[16,17]) (Figure 2).

Figure 2: Examples of polyketides



Doxorubicin

Avermectin B1b

Amphotericin B

Compactin

Spinosyn A

Erythromycin A

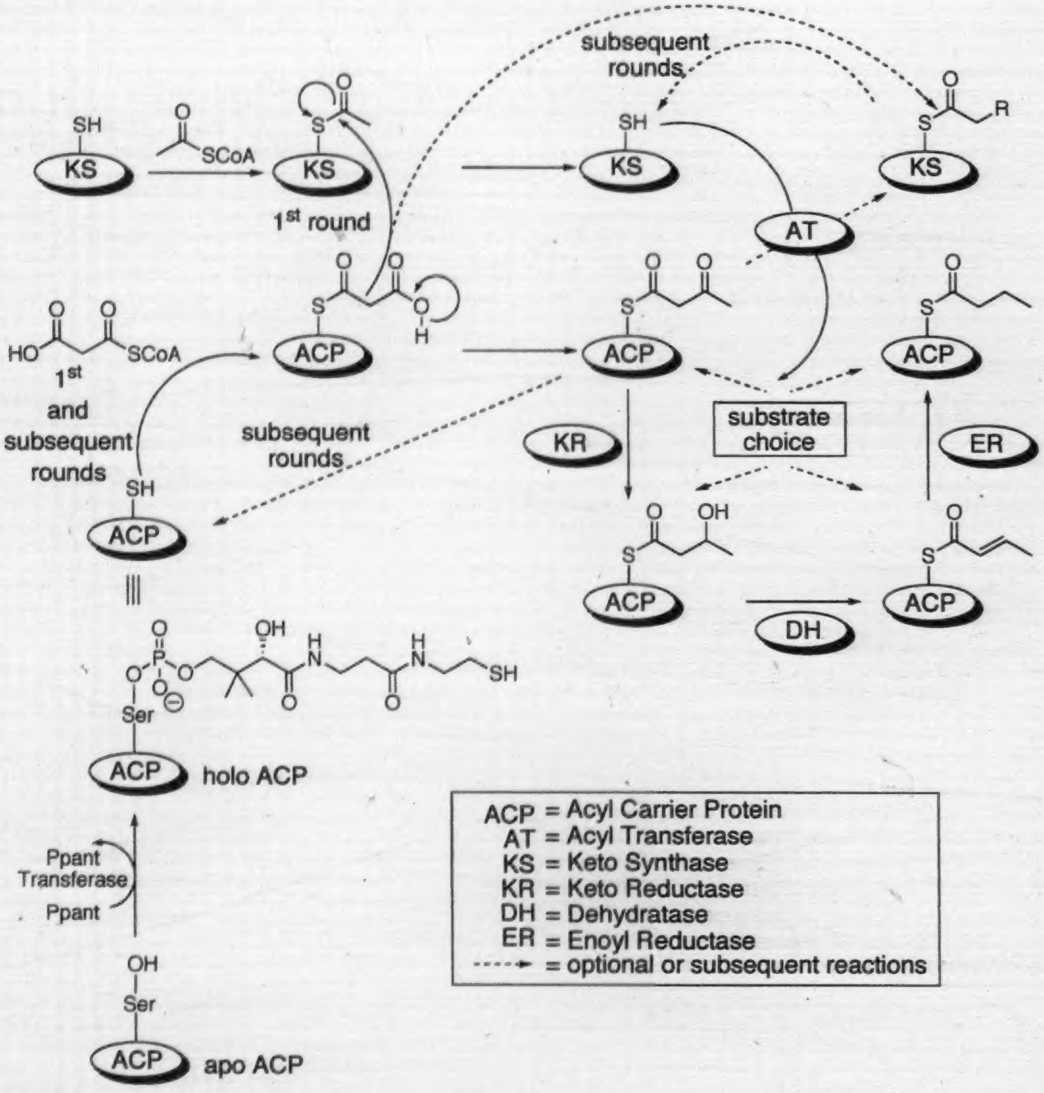
Rapamycin

1.1 Polyketide Biosynthesis

The biosynthesis of polyketides has been studied for a long time and continues to be actively investigated, with the hope that through the understanding of these pathways, new, novel, and more potent drugs will be developed.[1-6,18-27] Polyketides are synthesized by large multifunctional PKSs of which there are many classes: type I modular (bacteria),[3,4] iterative type I (fungi), iterative type II (bacteria) and type III (plants).[2] The various PKSs share some commonality, they all contain a minimum of three domains: an acyltransferase (AT), an acyl carrier protein (ACP) and a ketosynthase (KS). These domains are responsible for the loading of the starter units (typically a coenzyme A thioester such as acetyl-CoA or malonyl-CoA) and condensation to form the polyketide. In addition to these domains the PKSs can also contain ketoreductase (KR), dehydratase (DH), enoyl reductase (ER), thioesterase (TE) and methyltransferase (MeT) domains to yield various oxidation states of the product.

As mentioned above, fatty acid units in polyketides are assembled in a head to tail fashion. The most common starting materials for polyketides are acetyl-CoA and malonyl-CoA. These are loaded onto the respective domains (acetyl-CoA onto the KS and malonyl-CoA onto the ACP) by a transesterification from the CoA thioester onto either the active site cysteine of the KS or to the phosphopantetheinyl thiol found on the ACP. The phosphopantetheinyl (Ppant) group is a linker that activates a conserved serine on the apo (inactive) ACP converting it to the holo (active) ACP that can now be loaded with starting material. The Ppant group is used by the enzymes to shuttle the growing polyketide chain between the different domains of the PKS. During the first extension, the malonyl loaded ACP undergoes a decarboxylative condensation onto the acetyl loaded KS thereby yielding the free thiol of the KS and a ß-ketoacyl-*S*-ACP. This extended polyketide can either be transferred back to the KS by the AT domain and the ACP loaded with another malonyl unit for subsequent extension, or it can be acted upon by one or more of the KR, DH, ER and MeT domains before being transferred back to the KS for another round of extension (Figure 3).

Figure 3: General mechanism for polyketide extension

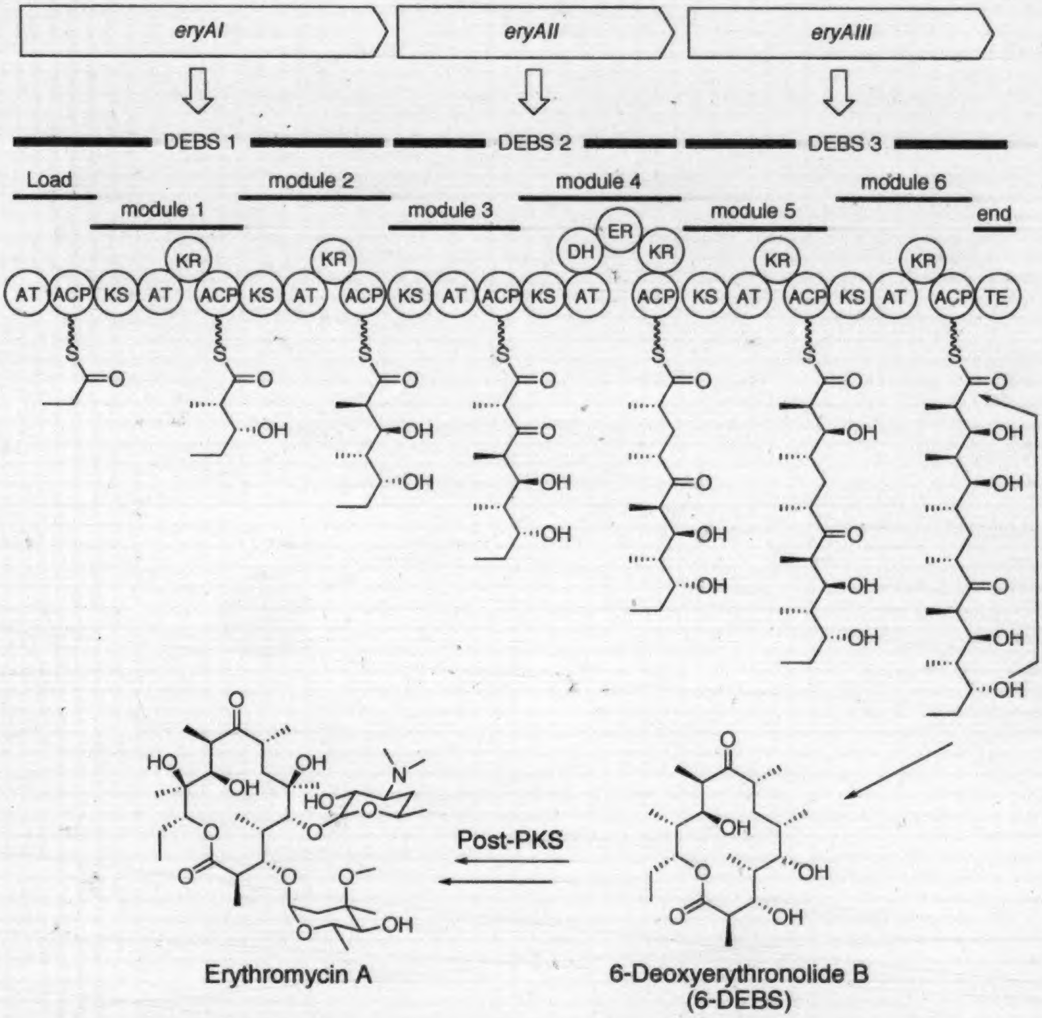

subsequent rounds
SH
KS
SCoA
KS
1st round
SH
KS
R
KS
AT
HO
SCoA
1st and subsequent rounds
ACP
H
ACP
ACP
substrate choice
KR
ER
subsequent rounds
SH
ACP
OH
ACP
ACP
DH
OH
Ser
SH
ACP
holo ACP
Ppant Transferase
Ppant
OH
Ser
ACP
apo ACP

ACP = Acyl Carrier Protein
AT = Acyl Transferase
KS = Keto Synthase
KR = Keto Reductase
DH = Dehydratase
ER = Enoyl Reductase
----► = optional or subsequent reactions

Modular PKSs have each of these domains located on individual proteins (module). Each of these modules is used only once, and in sequence. In many cases, the sequence of the domains appears to determine the final product of the PKS. In iterative PKSs, the domains are generally located on one large multifunctional protein (a single module) and the domains are used repeatedly in an iterative manner to make the final product. Prediction of the final product from the protein structure is not possible at present.

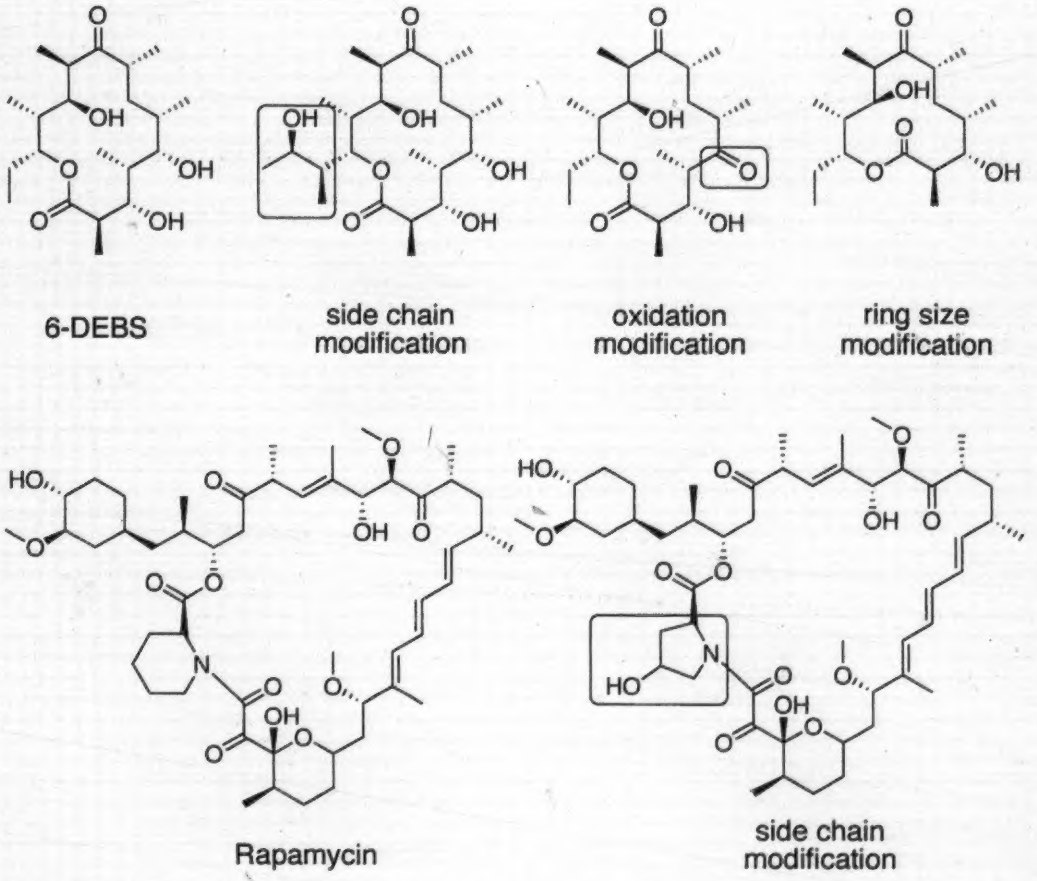
The extension process continues until the growing chain reaches the last module where it is released from the enzyme. This can occur by the intermolecular attack of water or by the action of a TE domain yielding a macrocycle through internal cyclization. The most studied modular PKS is the one producing erythromycin (Figure 4). In this system, 6-deoxyerythronolide B is produced by the PKS 6-deoxyerythronolide B synthase (DEBS). The cyclized product is then further elaborated in a series of post-PKS steps to erythromycin.[4]

Figure 4: Biosynthesis of 6-deoxyerythronolide B by the modular PKS (DEBS)


eryAI
eryAII
eryAIII
DEBS 1
DEBS 2
DEBS 3
Load
module 1
module 2
module 3
module 4
module 5
module 6
end
AT
ACP
KS
KR
DH
ER
TE
S
O
OH
Post-PKS
HO
N
Erythromycin A
6-Deoxyerythronolide B
(6-DEBS)

Modular PKSs have been widely studied. Through genetic manipulation, it is possible to generate new "non-natural" polyketides.[28-30] One of the most common ways to manipulate modular PKSs is to modify individual domains within a module by inactivation, deletion, or swapping. For example, inactivation of the KS domain, which starts the biosynthesis, followed by supplying the enzymes with non-natural di or triketides, can generate a new non-natural polyketide with changes at the starting units.[31-34] Switching the AT from another PKS is also possible and allows for additional variety of products.[35-38] Removing a domain from the assembly line can yield a different oxidation state at that position in the product.[39] It is also possible to modify the chain length of the polyketide by relocating the TE domain.[29,40,41] Manipulation of the domains can generate novel polyketides (Figure 5), but mutations often decrease the titer of the desired polyketide and limit the use for large scale production.[42,43]

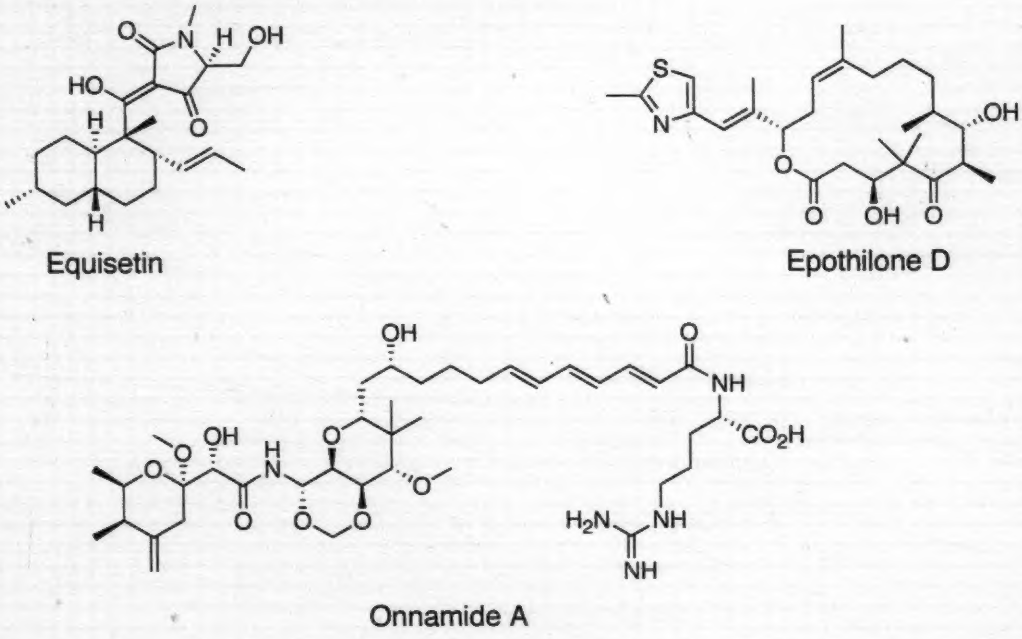
Figure 5: Novel polyketides prepared through genetic manipulation to produce side chain modifications of 6-DEBS[41] and rapamycin,[34] as well as oxidation level[39] and ring size[40] of 6-DEBS.


6-DEBS
side chain modification
oxidation modification
ring size modification
Rapamycin
side chain modification

Another approach to generating novel compounds is to substitute, add and/or remove whole modules. For example modules from two different PKSs (DEBS and rapamycin synthase) have been expressed together to produce a new polyketide.[44] Through study of this and other similar systems it is clear that for effective communication between the two non-native modules, it is necessary to manipulate their junction correctly.[44,45]

Non-ribosomal peptide synthetases (NRPSs) work in a very similar way to modular PKSs to make polypeptides, in that they are comprised of linked modules and the growing peptide chains are also linked to the enzyme *via* a Ppant arm.[46] Mixed PKS-NRPS systems already exist in nature[47] such as exemplified by onnamide A[48], equisetin[49] and epothilones[50] (Figure 6). With improved understanding, it may be possible to generate new mixed non-natural PKS-NRPS structures with valuable biological acticity.

Figure 6: Examples of mixed PKS-NRPS structures



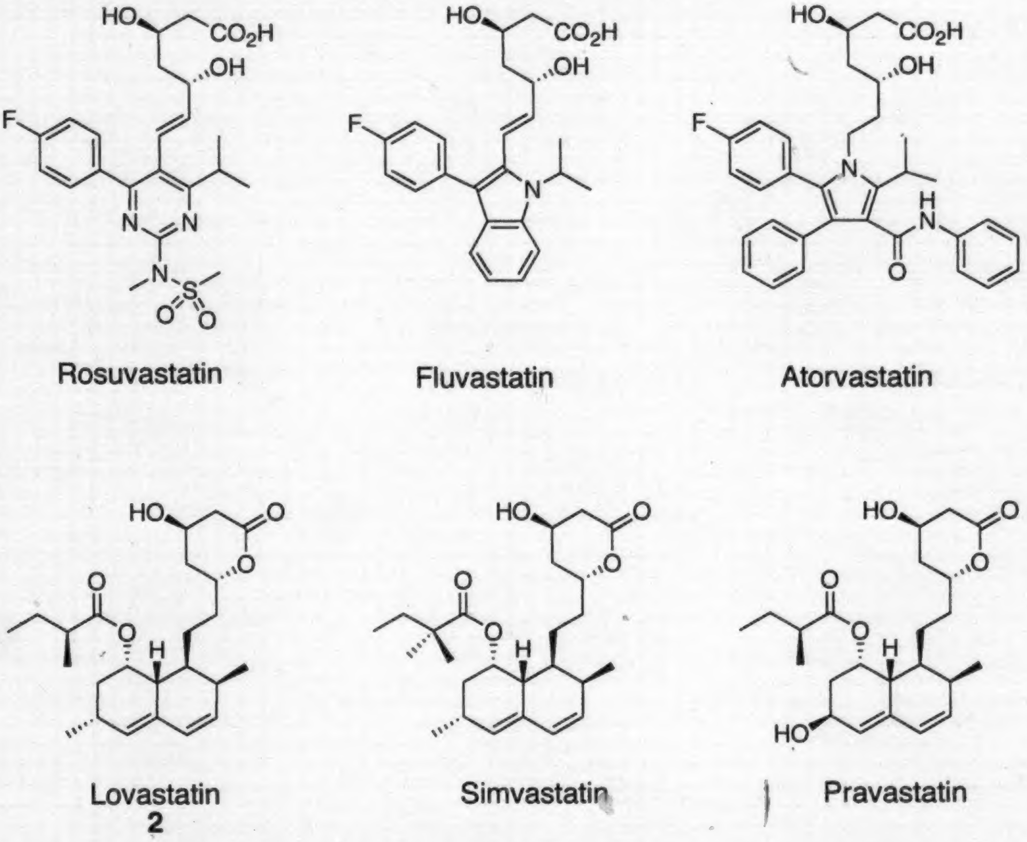
Iterative type I PKSs (which produce polyketides such as fumonisins,[51] equisetin[49] and lovastatin (**2**)[52,53]) are similar to the PKSs just described in that they contain the same overall set of domains and loading functions. However, that is where the similarities end. The iterative type I enzymes do not have multiple modules with individual domains that process the growing polyketide chain. Instead, as the name implies, a single set of active domains is used repeatedly to complete the biosynthesis of the final product. The mechanism by which these enzymes control functionality in the final product is not understood. Rather than the sequence of modules and domains dictating the structure of the final product, it appears that with the iterative type I systems, the enzyme bound intermediates along the biosynthetic pathway control which processing domains of the enzyme will be active. This fact makes it very difficult to manipulate any of the domains or active sites as the outcome would be unpredictable and could easily abolish any activity. A successful example is the modification to the MeT domain of *FUM1* (the PKS responsible for the biosynthesis of fumonisin mycotoxins) through a point mutation in the active site of the MeT, which generated a desmethyl analog of the fumonisin.[51]

Post-PKS steps occur on many polyketides and often enhance their biological activity. These steps can include hydroxylation and glycosylation, as in the case of the transformation of DEBS to erythromycin A (Figure 4), as well as methylation (on oxygen, nitrogen and/or carbon), epoxidation and dehydration.

1.2 Statins and Inhibition of Cholesterol Biosynthesis

As with many polyketides, the statins (Figure 7) are also of clinical relevance. Lovastatin (**2**) was the first statin to market in 1987 and was initially discovered for its antifungal activity from *Monascus ruber*[15] and *Aspergillus terreus*.[54] It was quickly realized to be a cholesterol-lowering agent due to the inhibition of (3*S*)-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase.[55]

Figure 7: Structure of natural, semi-synthetic and synthetic statins


Rosuvastatin

Fluvastatin

Atorvastatin

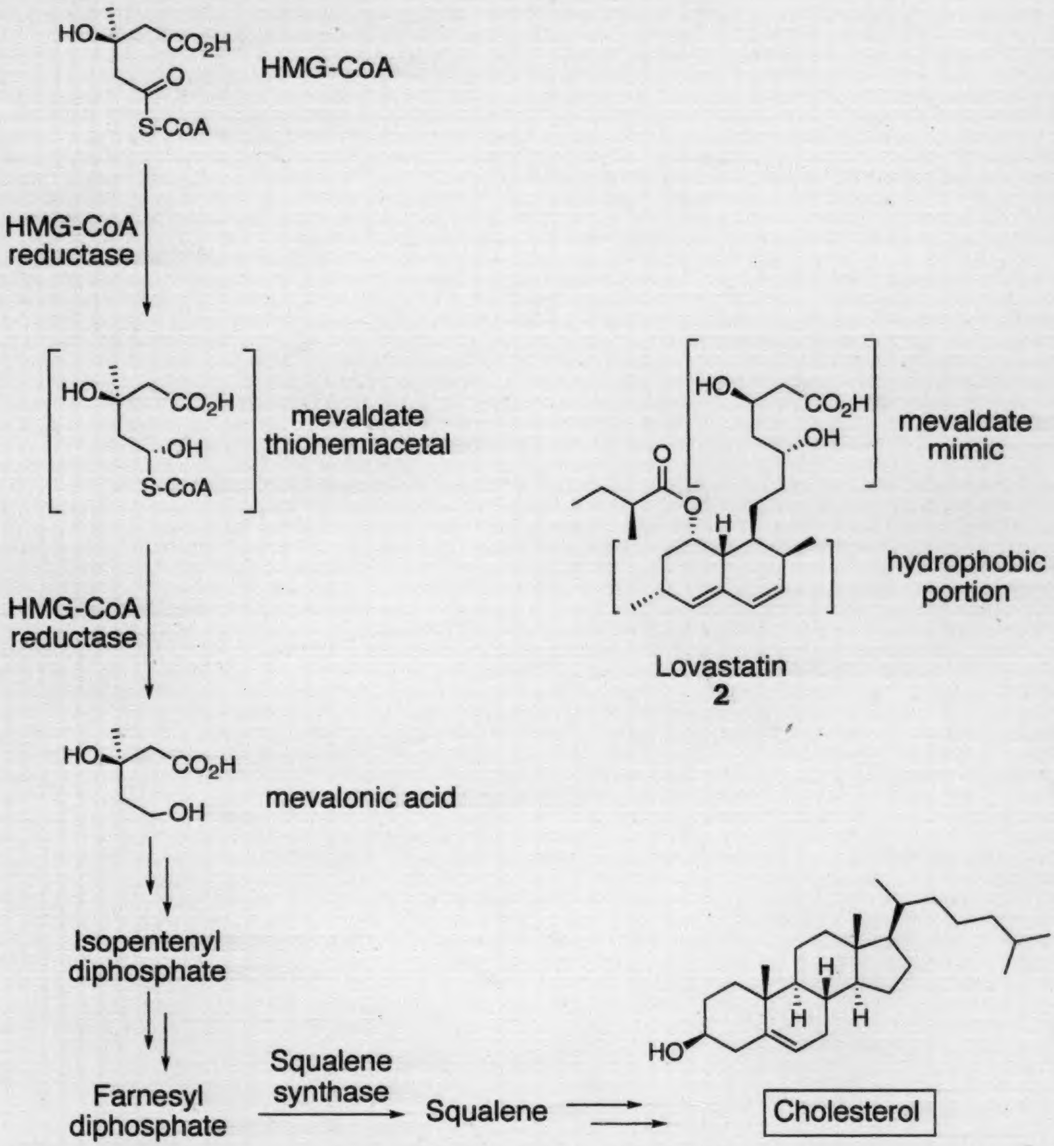
Lovastatin
2

Simvastatin

Pravastatin

The enzyme HMG-CoA reductase is responsible for the catalysis of the first committed step in the biosynthesis of cholesterol. The open lactone forms of the statins, including lovastatin (**2**), mimic the mevaldate thiohemiacetal intermediate in the transformation of HMG-CoA to mevalonate (Figure 8). Modeling studies with the crystal structure of HMG-CoA reductase indicate that all statins, which share the upper lactone ring, bind in the HMG-CoA binding site with the open form of the lactone. All statins also have a hydrophobic moiety (decalin ring for compactin, lovastatin (**2**) and simvastatin; fluorophenyl for atorvastatin, rosuvastatin and fluvastatin) that binds in a hydrophobic pocket of the active site. Additionally rosuvastatin and atorvastatin have polar functionalities (sulfone and amide groups respectively) which confer additional stability in the binding pocket through hydrogen bonding[56] that may also lead to their higher potency as cholesterol-lowering agents.

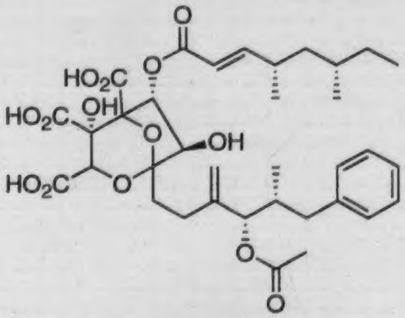
Figure 8: Cholesterol biosynthesis and its inhibition by lovastatin (**2**)


HMG-CoA
HMG-CoA reductase
mevaldate thiohemiacetal
HMG-CoA reductase
mevalonic acid
Isopentenyl diphosphate
Farnesyl diphosphate
Squalene synthase
Squalene
Cholesterol
mevaldate mimic
hydrophobic portion
Lovastatin
2

With the rise of cardiovascular disease in the developed world, the statins have become a leading therapy for the reduction of cholesterol. Atorvastatin and simvastatin were the two top selling drugs in the United States, with the statins as a class selling greater than 15 billion USD in 2004.[57] The statins are the primary drugs prescribed for reducing serum levels of cholesterol in humans. Their pharmacology has been extensively investigated.[58] Reduction of cholesterol levels by statins leads to an increase in the expression of the low-density lipoprotein (LDL) receptor, which results in increased catabolic degradation rate for plasma LDL. The statins are also able to reduce the levels of very low-density lipoproteins (VLDL), apolipoproteins, and thus triglycerides and LDL in the blood which is beneficial for the cardiovascular system. It is also proposed that statins have anti-inflammatory effects.[59] The statins also increase the levels of high-density lipoproteins (HDL) in the blood. Despite these advantages, there are some negative side effects. These include headaches, nausea, myopathy and rhabdomyolysis (which can be fatal).[58] Most of the statins are metabolized by oxidation, often by the microsomal cytochrome enzymes.[60] For example, lovastatin (**2**) and simvastatin are oxidized by cytochrome P_{450} CYP3A4 at C-6, which makes them more water soluble and easier to excrete. This is a common cytochrome enzyme responsible for the degradation of many metabolites. This enzyme is inactivated by a furanocoumarin (dihydroxybergamottin) found in grapefruit juice, and leads to increased serum levels of the drug if both are taken together. This can increase the probability of the negative side effects.[58]

Other methods for lowering cholesterol levels include the use of a combination of drugs such as simvastatin with ezetimibe, a cholesterol absorption inhibitor, called vytorin.™ Inhibition of HMG-CoA reductase with statins is an early step in the biosynthesis of cholesterol, and thus there is also a concomitant reduction in the biosynthesis of other important isoprenoids, such as farnesyl and geranylgeranyl diphosphate. It might therefore be more advantageous to inhibit a later stage in the biosynthesis. A possible target would be the inhibition of squalene synthase which might be accomplished with the potent polyketide inhibitor squalestatin S1[18] (Figure 9).

Figure 9: Structure of the squalene synthase inhibitor squalestatin S1

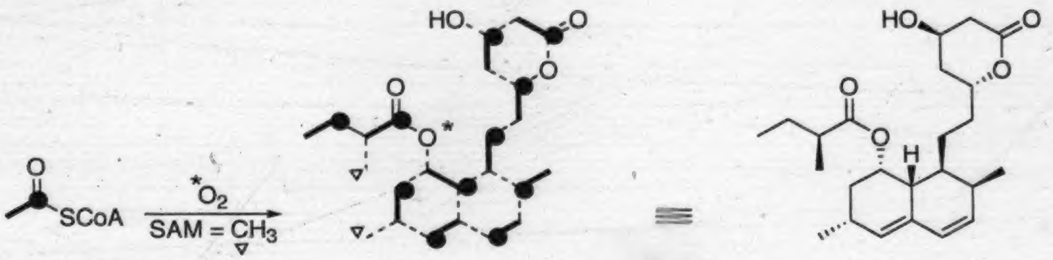


Squalestatin S1
an inhibitor of
squalene synthase

1.3 Lovastatin Biosynthesis and its Enzymes

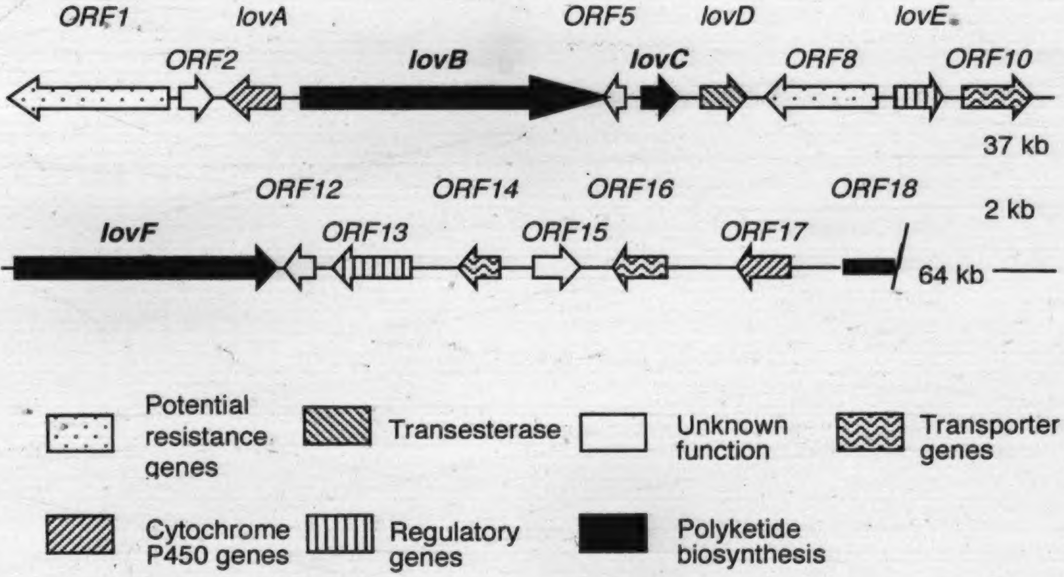
Due to the importance of the statins and their interesting structure, a significant effort has been made to understand their biosynthesis.[61-67] The structural similarities between compactin and lovastatin (**2**) indicate their biosynthetic pathways are very similar,[68] however only the biosynthesis of lovastatin (**2**) will be discussed. Lovastatin (**2**) is made of two polyketide chains. A nonaketide (1 acetate and 8 malonate units) produced by the lovastatin nonaketide synthase (LNKS) and a diketide side chain (1 acetate and 1 malonate unit) produced by the lovastatin diketide synthase (LDKS). It has one oxygen derived from molecular oxygen at C-8 and 2 methyl groups at C-6 of the decalin ring and C-2 of the butyryl side chain from *S*-adenosyl methionine (SAM) (Figure 10).

Figure 10: Molecular origins of lovastatin (**2**)


SCoA
*O2
SAM = CH3
▽
HO
O
O
O*
H

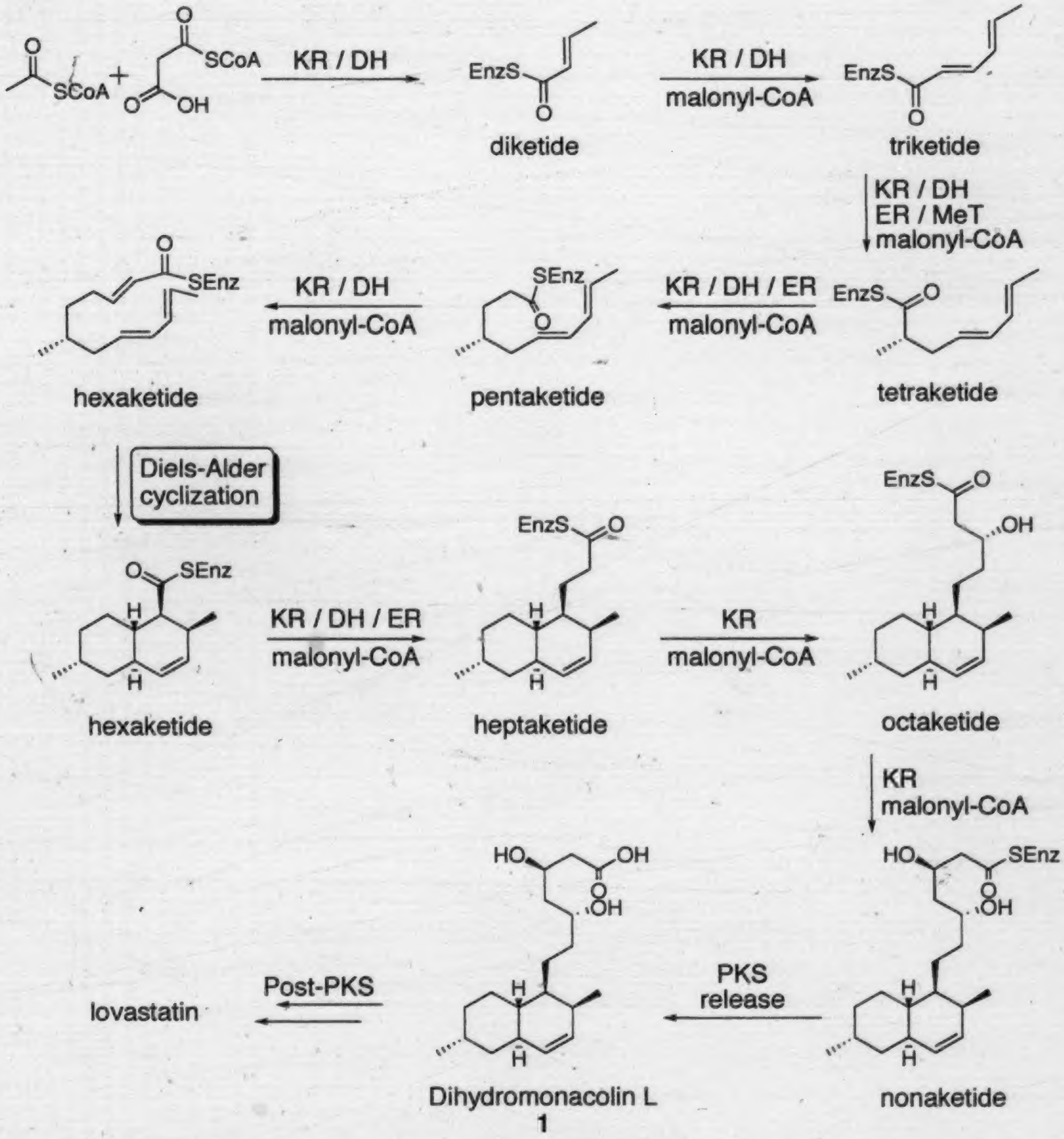
Lovastatin
2

Figure 11: Lovastatin biosynthetic gene cluster[52]


ORF1
lovA
ORF5
lovD
lovE
ORF2
lovB
lovC
ORF8
ORF10
37 kb
ORF12
ORF14
ORF16
ORF18
2 kb
lovF
ORF13
ORF15
ORF17
64 kb
Potential resistance genes
Transesterase
Unknown function
Transporter genes
Cytochrome P450 genes
Regulatory genes
Polyketide biosynthesis

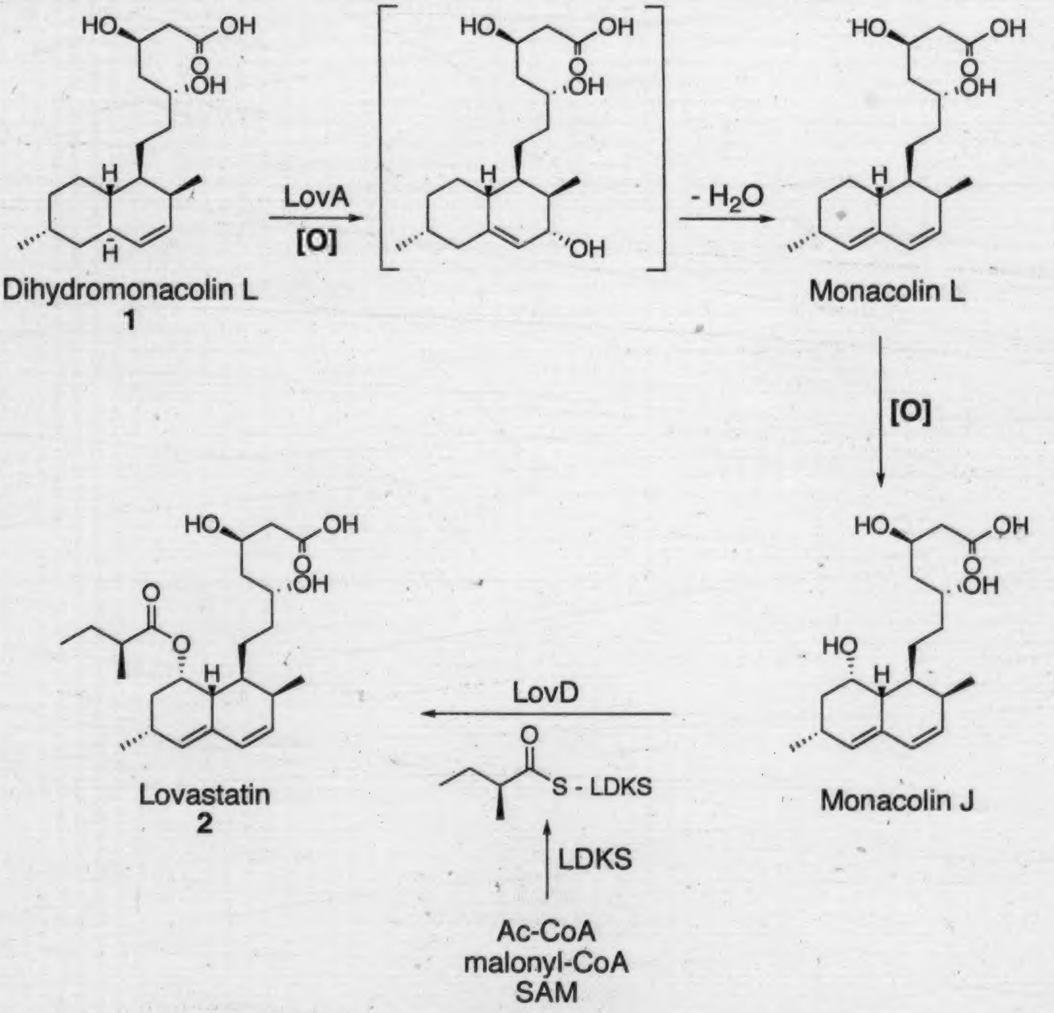
Genetic research revealed a cluster of 18 genes related to lovastatin biosynthesis (Figure 11).[52,53] Through genetic manipulation it has been determined that **1** is produced from the action of two enzymes, namely LNKS (LovB) and an accessory protein (LovC). The first released intermediate along the proposed biosynthetic pathway is dihydromonacolin L (**1**) (Figure 12).

Figure 12: Proposed biosynthetic pathway of dihydromonacolin L (1) by LovB and LovC


SCoA
SCoA
OH
KR / DH
EnzS
diketide
KR / DH
malonyl-CoA
triketide
KR / DH
ER / MeT
malonyl-CoA
tetraketide
KR / DH / ER
malonyl-CoA
SEnz
pentaketide
KR / DH
malonyl-CoA
hexaketide
Diels-Alder
cyclization
hexaketide
KR / DH / ER
malonyl-CoA
heptaketide
KR
malonyl-CoA
octaketide
KR
malonyl-CoA
nonaketide
PKS
release
Dihydromonacolin L
1
Post-PKS
lovastatin

Once dihydromonacolin L (**1**) is produced by the LovB and LovC enzymes, it undergoes a series of post-PKS steps to make lovastatin (**2**). The first proposed step is the oxidation at C-3 by the gene product of *lovA* that has homology with known cytochrome P_{450}. The allylic alcohol is thought to spontaneously dehydrate forming the diene monacolin L, although this step has yet to be proven and is thought by workers at Merck™ to be an artifact of isolation.[69] This step is being investigated in our group by Mr. Jamie Côté. Oxidation at C-8, producing monacolin J, followed by transesterification with the LDKS-bound diketide using the LovD enzyme yields lovastatin (**2**) (Figure 13).

Figure 13: Post-PKS steps, from dihydromonacolin L (**1**) to lovastatin (**2**)

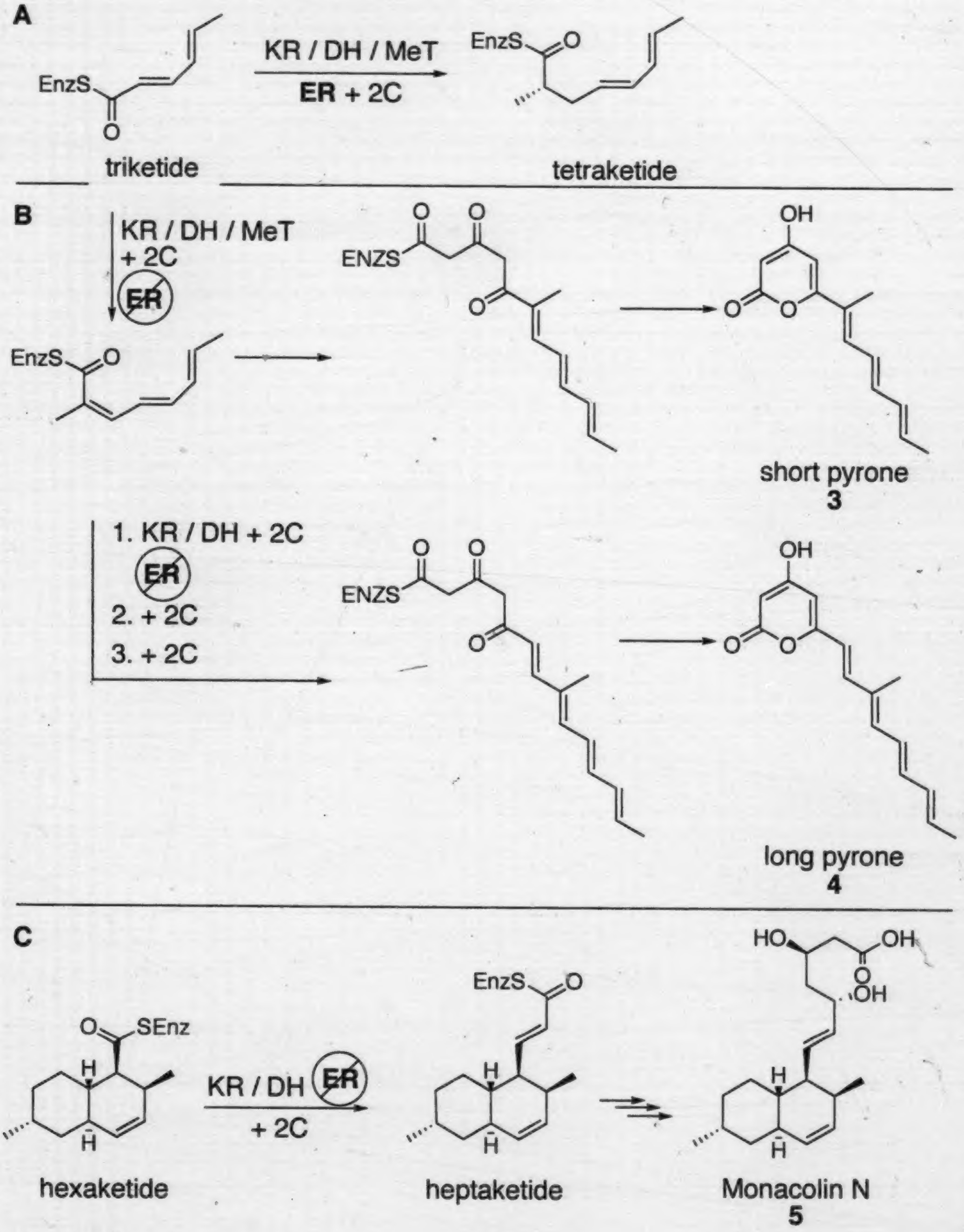


HO
OH
O
OH
H
H
Dihydromonacolin L
1
LovA
[O]
OH
- H2O
Monacolin L
[O]
Monacolin J
LovD
S - LDKS
LDKS
Ac-CoA
malonyl-CoA
SAM
Lovastatin
2

The LNKS has one set of all the domains, AT, ACP, KS, KR, DH, MeT, and ER necessary for the complete biosynthesis. However, if this large enzyme (335 kDa) is expressed in the heterologous host *A. nidulans*, no dihydromonacolin L (**1**) is produced.

Instead two new shunt metabolites **3** and **4** are isolated (Figure 14).[52] These pyrones (originating from 6 or 7 acetate units and one unit of SAM each) are also isolated from mutants that have had *lovC* inactivated. The LovC protein has high similarity with known PKS ER domains, and the shunt products indicate that the ER domain of LNKS is not functioning correctly in going from the triketide to tetraketide stage. This step requires the action of LovC either as an ER or to modulate the activity of the LNKS ER. Without LovC present it appears that LovB can accomplish the initial stages of the proposed pathway including use of the MeT domain, but fails to recognize the substrate correctly once the ER step is not performed. Co-expression of *lovB* and *lovC* in *A. nidulans* produces **1** indicating that only these two enzymes are required to make the nonaketide. It is curious to note that another failure in this LovB+C system has been discovered that generates monacolin N (**5**).[70] Compound **5** has an extra degree of unsaturation between the decalin and lactone rings of dihydromonacolin L (**1**) that appears due the failure of LovC to reduce the heptaketide intermediate. However, LovB in this case is able to complete the synthesis. It is interesting that the location of the extra degree of unsaturation is the same as in the synthetic statins, fluvastatin and rosuvastatin.

Figure 14: Enoyl reductase (LovC) failure. **A**: Normal enoyl reductase activity. **B**: Enoyl reductase failure generating the shunt metabolites (short pyrone **3** and long pyrone **4**). **C**: Enoyl reductase failure producing monacolin N (**5**).


A
EnzS
O
triketide
KR / DH / MeT
ER + 2C
EnzS
O
tetraketide
B
KR / DH / MeT
+ 2C
ER
EnzS
O
ENZS
O
O
O
OH
O
O
short pyrone
3
1. KR / DH + 2C
ER
2. + 2C
3. + 2C
ENZS
O
O
O
OH
O
O
long pyrone
4
C
O
SEnz
H
H
hexaketide
KR / DH
ER
+ 2C
EnzS
O
H
H
heptaketide
HO
OH
O
OH
H
H
Monacolin N
5

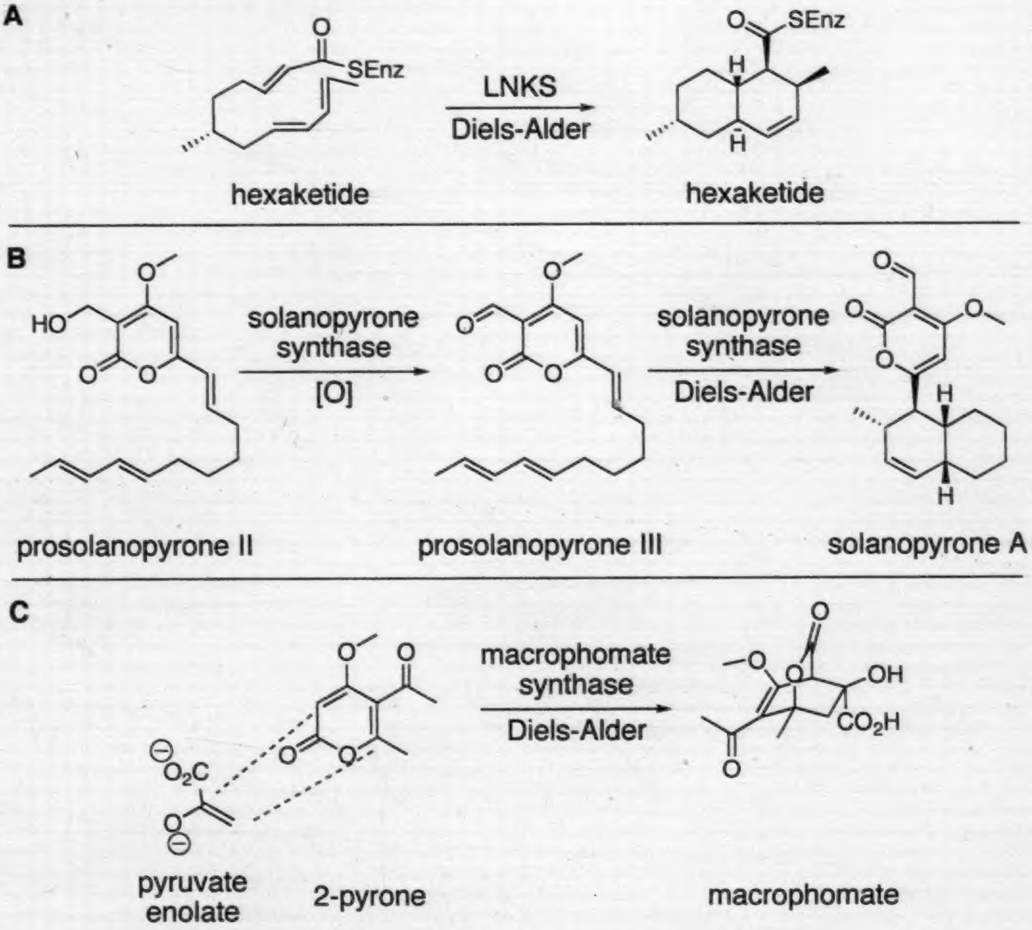
To better understand the biosynthetic pathway leading to dihydromonacolin L (**1**) as well as the loading capabilities of the AT and ACP, we would like to incorporate advanced precursors using the purified enzyme. If they are accepted and carried through to **1**, it would indicate that they are along the biosynthetic pathway and that the LNKS loading domain is a little promiscuous. It has been shown that NAC thioesters mimic natural CoA thioesters for incorporation into PKS systems.[71] Another system that has been accepted by the loading domain of PKSs are thioglycolates.[28] A method that has been used to incorporate precursors has been to link the intermediate to an ACP domain chemically and have the ACP transfer it to the KS domain.[72] Initial *in vivo* studies attempting to incorporate NAC thioesters of labeled hexaketide into lovastatin (**2**) have not met with success as they are degraded in the media very quickly.[73] For incorporation to be successful, purified or semi-purified enzymes must be used. This aspect of the activity will be investigated through the synthesis of advanced intermediates and use of purified enzymes.

LNKS is known to produce the pyrones **3** and **4** *in vivo* and has been proposed to generate them *in vitro* as seen by UV assays at 365 nm upon addition of the substrates [malonyl-CoA, Ac-CoA, β-nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD) and SAM] to purified enzymes. The pyrones have yet to be isolated in such *in vitro* assays. Another goal of this thesis is to synthesize the pyrones **3** and **4** and their saturated derivatives as standards for HPLC analysis of enzymatic assays.

1.4 Diels-Alderases

During the biosynthesis of dihydromonacolin L (**1**) by LovB and LovC the proposed hexaketide intermediate (Figure 12) is thought to undergo a Diels-Alder reaction while still bound to the enzyme.[52] The Diels-Alder reaction is a [4+2] cycloaddition involving a 1,3-diene and a dienophile whose π-orbitals overlap, making two new carbon-carbon bonds in a concerted manner (not necessarily synchronous), to generate a new 6-membered ring.[74] Nature uses this reaction to make a variety of novel structures with a high degree of stereoselectivity to produce multiple stereogenic centers in one reaction.[75,76] To date only three possible Diels-Alderase enzymes have been documented (Figure 15). One being the LNKS,[77] the second being solanopyrone synthase[78] and the third being macrophomate synthase[79] (Figure 15). Solanopyrone synthase has not been obtained in pure form, but an x-ray crystal structure of macrophomate synthase has been reported.[80] However, recent theoretical calculations have indicated that the reaction catalyzed by macrophomate synthase is a non-concerted series of anionic condensations.[81]

Figure 15: Reactions of natural putative Diels-Alderases. **A**: LNKS. **B**: Solanopyrone synthase. **C**: Macrophomate synthase.


A
O
SEnz
LNKS
Diels-Alder
O
SEnz
H
H
hexaketide
hexaketide
B
HO
O
O
O
solanopyrone synthase
[O]
O
O
O
O
solanopyrone synthase
Diels-Alder
O
O
O
O
H
H
prosolanopyrone II
prosolanopyrone III
solanopyrone A
C
O
O
O
O
O₂C
O
macrophomate synthase
Diels-Alder
O
O
OH
CO₂H
O
pyruvate enolate
2-pyrone
macrophomate

A number of artificial Diels-Alderases have been generated in the form of catalytic antibodies[82,83] that lower the activation barrier of the reaction by holding the intermediates in a tight binding pocket. Yet another method of catalyzing a Diels-Alder reaction is through the use of ribozymes.[84]

One of the goals of this project is to study the mechanism of the Diels-Alderase reaction catalyzed by LNKS by determining the secondary deuterium kinetic isotope effect (KIE). This is a standard method for distinguishing between a concerted and stepwise mechanism.[85-92] We envision doing this by synthesizing deuterated analogs of the hexaketide triene NAC thioester and individually competing these with the unlabeled analog in the active site of purified LNKS and analyzing the cyclized products by GC-MS.[93]

The objectives of this thesis are three fold regarding the *in vitro* activity of the lovastatin nonaketide synthase (LNKS): to investigate the Diels-Alderase activity using secondary deuterium kinetic isotope effects, to explore the enzyme activity by a UV assay for the production of the pyrones and to study the biosynthetic intermediates of dihydromonacolin L (**1**).

CHAPTER 2. Results and Discussion

2.1 Incorporation assays to further understand the biosynthetic pathway of dihydromonacolin L (1)

Determining the exact biosynthetic pathway of natural products is a challenging undertaking. One very important aspect is to know the origins of the atoms. This is typically done using isotopic labeling studies. Small molecules such as acetate, oxygen gas and amino acids are isotopically labeled, and these are used as sources of carbon, hydrogen, nitrogen and oxygen for the organism producing the natural product. The origins of the atoms and intact bonds derived from primary metabolites has been determined for lovastatin (**2**) (Figure 10).[61-66] The genes responsible for producing the proteins that generate bacterial and fungal natural products are often clustered together[94] and can be incorporated into another host that does not normally produce the target metabolite.[52] It is also possible to knock out or delete certain genes and therefore their gene products. This may cause accumulation of an intermediate along the biosynthetic pathway, thereby identifying it. It may also permit addition of putative precursors past the block to determine the intermediates in the later stages of the biosynthesis. The above-mentioned methods have been used to obtain a large amount of information about the biosynthetic pathway of lovastatin (**2**) and dihydromonacolin L (**1**).[52,61-63,77,95] An additional method, which helps to define the intermediates along the pathway, is to feed proposed labeled precursors to the organism in the hope that they will be transformed to the natural product. Incorporation of late stage precursors into the pathway has proved successful using *in vivo* studies with *Aspergillus terreus* mutants.[95] For example a *lovC* mutant is able to convert dihydromonacolin L (**1**) and monacolin J into lovastatin (**2**)

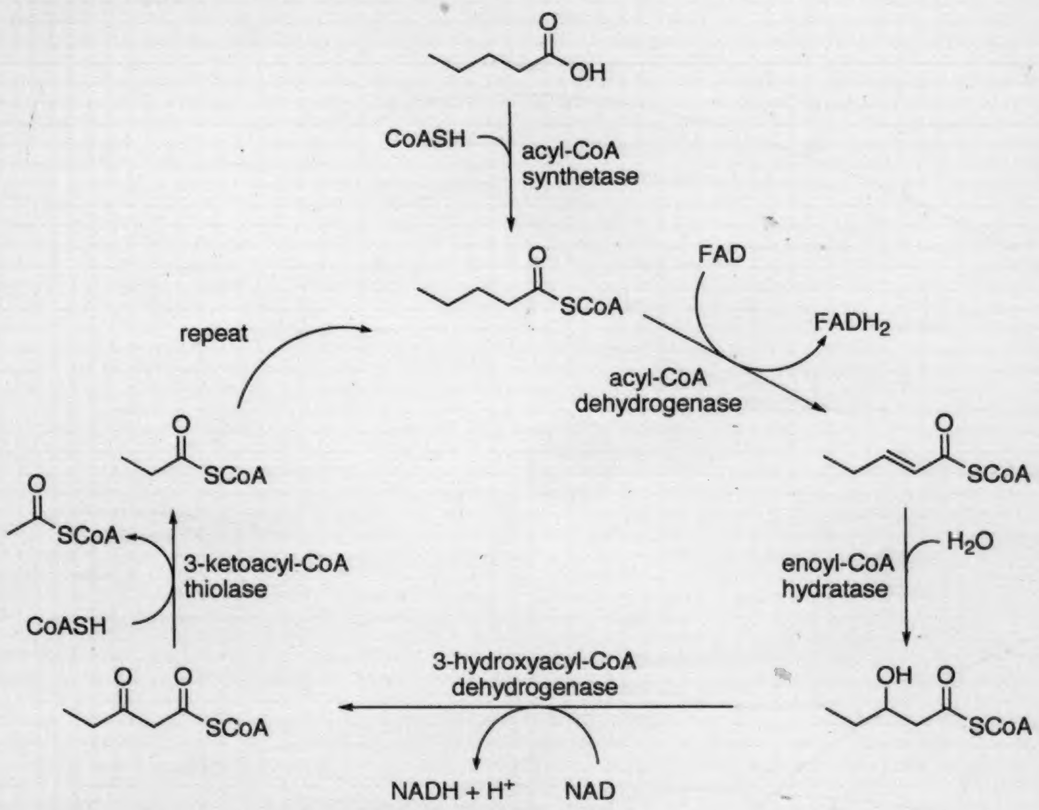
(Figure 13).[95] This indicates that the labeled compound is probably an intermediate in the biosynthesis. To date attempts to incorporate partially assembled polyketides using fungal cultures has not yielded **2**, as such precursors are rapidly degraded.[73]

Table 1: Genetically engineered organisms used during this work

Organism and genes	**Enzymes produced**	**Description**
A. nidulans *lovB*	lovastatin nonaketide synthase (LNKS)=(LovB)	*lov*B gene from *A. terreus* expressed heterologously in *A. nidulans.*
A. nidulans *lovC*	putative enoyl reducase (LovC)	*lov*C gene from *A. terreus* expressed heterologously in *A. nidulans.*
A. nidulans *lovB+C*	LovB and LovC	*lovC* gene from *A. terreus* expressed heterologously in *A. nidulans lovB*

One goal of this thesis is to investigate the early stages of the biosynthesis of dihydromonacolin L (**1**) and hence lovastatin (**2**) using purified enzymes. The use of purified enzymes should avoid the problems of rapid degradation of the precursors by ω- and β-oxidation (Scheme 1) systems or by hydrolysis of the thioester that is seen with *in vivo* systems. This should also avoid potential problems of transport of the intermediate through the cell wall prior to conversion by LNKS.

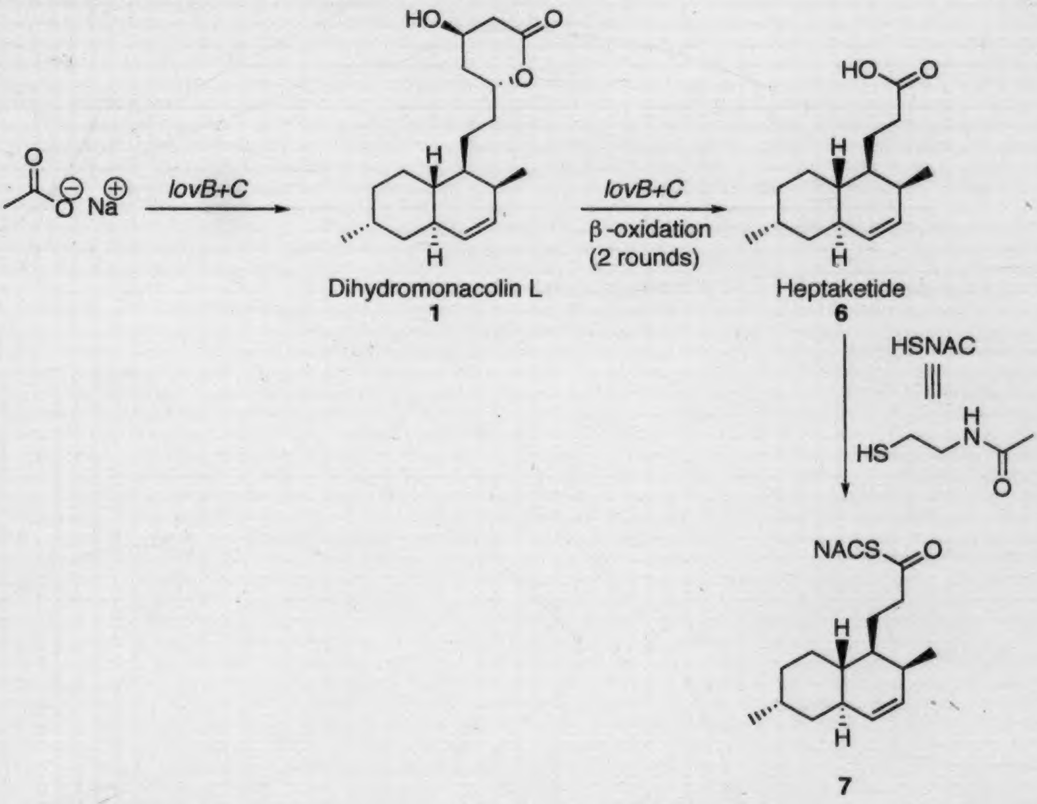
Scheme 1: β-oxidation of fatty acids and polyketides


OH
CoASH
acyl-CoA synthetase
SCoA
FAD
FADH2
acyl-CoA dehydrogenase
repeat
SCoA
SCoA
3-ketoacyl-CoA thiolase
CoASH
SCoA
enoyl-CoA hydratase
H2O
OH
SCoA
3-hydroxyacyl-CoA dehydrogenase
NADH + H+
NAD
SCoA

2.1.1 Synthesis of the heptaketide-NAC thioester 7

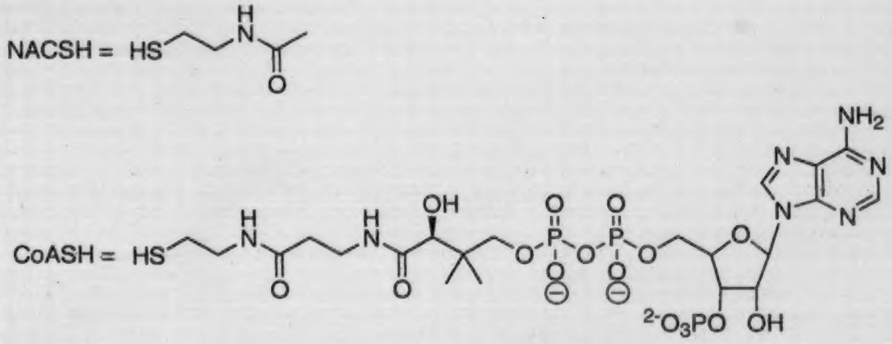
In order to better understand the proposed biosynthetic pathway of lovastatin (**2**), the heptaketide-NAC **7** ester was synthesized by Dr. Hiroyuki Morita.[96] Fermentation of *Aspergillus nidulans* containing heterologously expressed *lovB+C* genes, as described in the experimental section for the isolation of dihydromonacolin L (**1**), allowed isolation of the heptaketide acid **6**. The heptaketide **6** is probably derived from two rounds of β-oxidation of dihydromonacolin L (**1**) rather than *via* direct production by LovB+C. After addition of NaOAc to this organism for 5 days, the culture broth was extracted with CH_2Cl_2 and the crude extract was purified by flash chromatography to yield the heptaketide acid **6**. The acid **6** was then converted to the NAC thioester **7** through coupling with N-acetyl-cysteamine (Scheme 2).

Scheme 2: Synthesis of heptaketide NAC thioester **7**



NAC thioesters are known to be substrates for PKSs as they mimic CoA thioesters (Figure 16).[71,97] It is proposed that the enzyme-bound thioester of heptaketide **6** is on the biosynthetic pathway to the dihydromonacolin L (**1**).[52,98] If LNKS (LovB) is able to load **7** onto its ACP domain, then it should be able to carry **7** though to dihydromonacolin L (**1**) using the substrates malonyl-CoA and NADPH. Incorporation rates can be very low and thus radioactive malonyl-CoA is used in conjunction with unlabeled malonyl-CoA to allow detection of small amounts of transformation.

Figure 16: NAC mimics CoA



2.1.2 Studies on incorporation of heptaketide-NAC thioester 7 into dihydromonacolin L (1)

An active cell free extract (CFE) of LovB was prepared from the *A. nidulans lovB* strain (UAMH8965, WMH1738), that had been generously given to us by Professor Hutchinson from the University of Wisconsin-Madison (now with Kosan Biosciences),[52] using a modification of the method of Reeves and coworkers[53] described in the experimental section. This strain has the *lov*B gene from *A. terreus* expressed heterologously in *A. nidulans*. The LovB activity in this CFE was tested using a standard UV assay. An increase in absorption was observed at 360 nm when the substrates (Ac-CoA and malonyl-CoA) and the cofactors (NADPH, FAD and SAM) were added. The increase in absorption is proposed to be due to the production of the pyrones **3** and **4** by LovB. If the CFE was inactivated (boiled for 30 min) and all the substrates and cofactors were added, or if Ac-CoA and malonyl-CoA were absent, no increase in absorption was seen. As the LovB enzyme appeared active, the heptaketide NAC ester **7** in EtOH, FAD, NADPH and both radioactive and unlabeled malonyl-CoA were added and the reaction mixture was shaken on an orbital shaker for 20 h. Purification by HPLC revealed starting NAC ester **7** (80%) but dihydromonacolin L (**1**) was not observed.

A CFE of LovB was prepared using a simplified extraction buffer consisting only of phosphate buffer (5 mM) with dithiothreitol (DTT). This method also produced active LovB as determined by the UV assay. This active LovB was preincubated for 40 min with **7** prior to adding FAD, NADPH, and the radioactive malonyl-CoA. After 10 min unlabeled malonyl-CoA was added and the mixture shaken on an orbital shaker for 20 h. Purification revealed starting material **7** and hydrolyzed heptaketide **6**, but no **1** was observed.

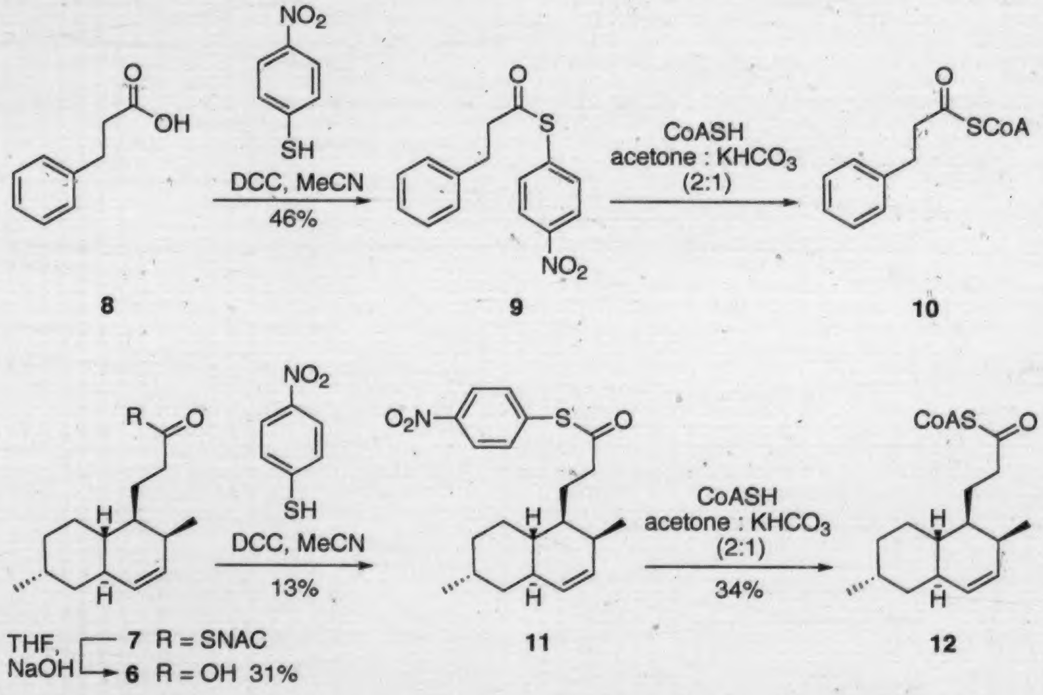
2.1.3 Studies on incorporation of heptaketide-NAC thioester 7 into dihydromonacolin L (1) using cell free extracts of *A. nidulans lovB+C*

There are a number of possible reasons why no dihydromonacolin L (**1**) was produced *in vitro* by LovB. It is known that the *A. nidulans lovB* clone generates a correct protein due to the fact that when the *lovC* gene is added to the *lovB* clone to give the *A. nidulans lovB+C* organism, it generates dihydromonacolin L. It is reasonable that LovC must be present to modulate the correct functioning of LovB. To test this proposition a CFE of LovB+C was prepared. FAD, NADPH, Ac-CoA and the heptaketide-NAC thioester **7** was added to the CFE along with a mixture of radiolabeled and unlabeled malonyl-CoA and the mixture shaken on an orbital shaker. An equivalent portion of **7** and the malonyl-CoA solution were added after 12 h. After a total time of 21 h, purification by HPLC revealed only starting material **7**, and no **1** could be detected.

2.1.4 Studies on incorporation of heptaketide-CoA thioester 12 into dihydromonacolin L (1)

It is also possible that LovB may require a CoA thioester for loading and synthesis initiation. To test this hypothesis the CoA thioester of the heptaketide was synthesized. CoA thioesters are known to be unstable. Many methods to prepare CoA esters are known.[99] Due to the value of the heptaketide, test reactions using 3-phenylpropionic acid (**8**) were performed to validate the synthetic plan. Initial attempts to activate 3-phenylpropionic acid as the mixed anhydride[100] with ethyl chloroformate (as monitored by ^{1}H-NMR and low resolution electrospray mass spectrometry (LRMS-ES)) followed by transesterification with CoASH failed to yield the desired product as determined by LRMS-ES. ^{1}H-NMR of the crude mixture indicates that the mixed anhydride is formed, but that the subsequent transesterification fails. Raising the pH from 8 to 9.5 during the transesterification did not give better results. Chase and Tubbs report the transthioesterification of a thiophenol thioester with CoASH.[101] To further activate the acid, a *p*-nitrothiophenol derivative **9** of 3-phenylpropionic acid (**8**) was prepared. This was then successfully transthioesterified with CoASH to yield **10** (Scheme 3). The heptaketide-CoA ester **12** was prepared using the same method from **7** (Scheme 3).

Scheme 3: Synthesis of CoA thioesters


NO2
OH
SH
DCC, MeCN
46%
S
NO2
CoASH
acetone : KHCO3
(2:1)
SCoA
8
9
10
NO2
R
O
SH
DCC, MeCN
13%
O2N
S
O
CoASH
acetone : KHCO3
(2:1)
34%
CoAS
O
H
H
THF, NaOH
7 R = SNAC
6 R = OH 31%
11
12

2.1.5 Studies on incorporation of heptaketide-CoA thioester 12 into dihydromonacolin L (1) using cell free extracts of *A. nidulans lovB*

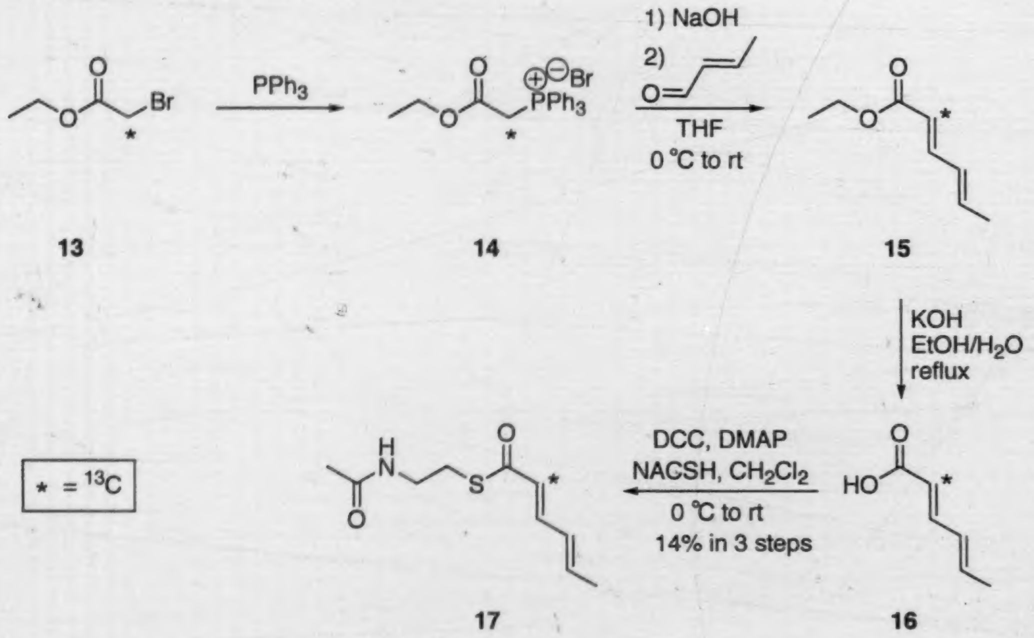
Incorporation of the heptaketide-CoA thioester **12** into **1** was attempted using an active CFE of LovB. Compound **12**, FAD and NADPH were incubated with the CFE for 10 min before pulse feeding a mixture of radiolabeled and unlabeled malonyl-CoA 10 times over 9 h. After quenching and extraction, part of the crude extract was purified by HPLC but **1** was not detected. To the remainder of the crude extract unlabeled product **1** was added in order to assist detection of minute amounts of labeled **1** that were possibly formed (radioactive dilution assay). The mixture was purified by HPLC, but the recovered **1** was not radioactive.

Attempted incorporation of the heptaketide thioesters **7** and **12** into dihydromonacolin L (1) to date has been unsuccessful. Possible explanations for this could be that the entrance to the active site of LovB does not allow for large molecules to enter. Another possible reason could be that the active site thiol is oxidized even though precautions were taken to avoid this (the use of DTT in the buffers). Another explanation may be that during the process of extracting the protein to form the CFE, one or more factors necessary for the function of LovB are removed.

2.1.6 Studies on incorporation of triketide-NAC thioester 17 into 1

To test the theory that the active site may not accept a molecule as large as the heptaketide, a smaller molecule proposed to be on the biosynthetic pathway was synthesized. The ^{13}C labeled triketide **17** was prepared by Mr. Kris Rathwell,[102] an undergraduate student in our laboratory (Scheme 4).

Scheme 4: Synthesis of ^{13}C labeled triketide


Br
PPh3
Br
PPh3
1) NaOH
2)
O
THF
0 °C to rt
13
14
15
KOH
EtOH/H2O
reflux
* = 13C
H
N
O
S
DCC, DMAP
NACSH, CH2Cl2
0 °C to rt
14% in 3 steps
HO
17
16

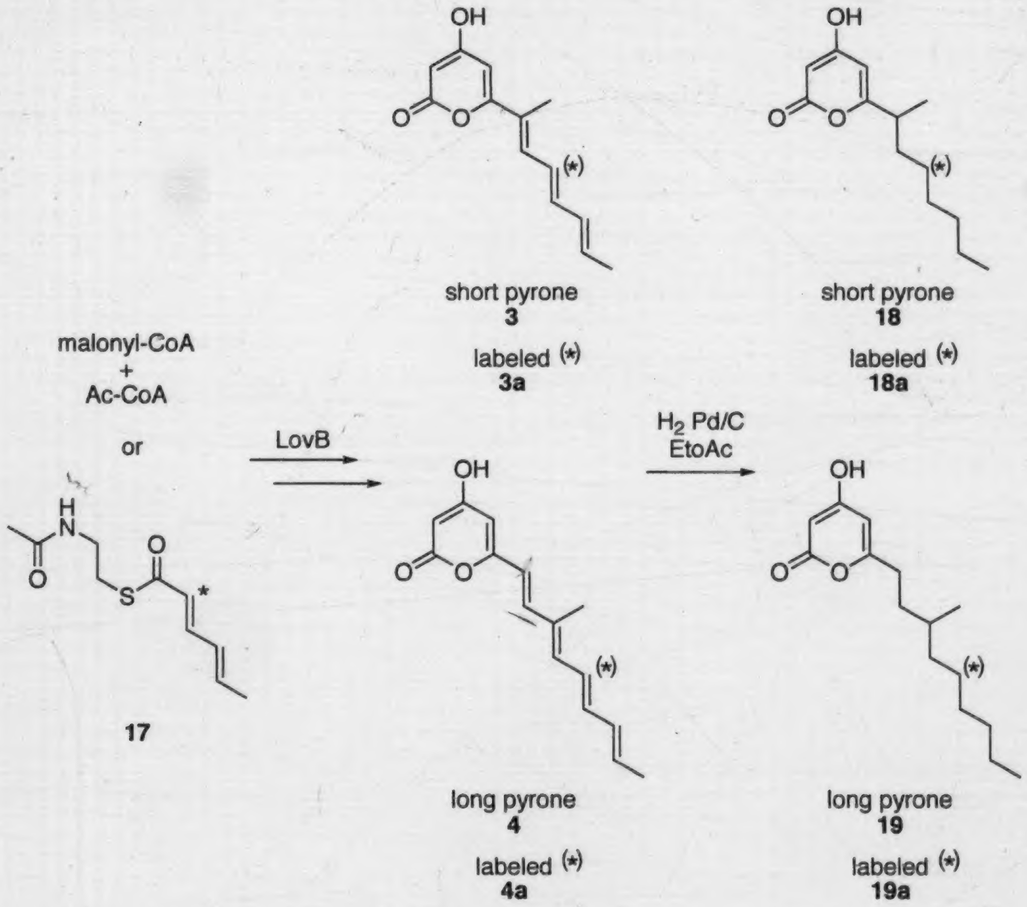
2.1.7 Studies on incorporation of ^{13}C labeled triketide NAC thioester 17 into dihydromonacolin L (1) using cell free extracts of *A. nidulans lovB+C*

An active CFE of LovB+C was prepared. The LovB+C should be able to convert **17** into dihydromonacolin L (**1**). The NAC ester **17** was incubated with the CFE for an hour before adding FAD, NADPH, SAM and malonyl-CoA and leaving the reaction mixture on the orbital shaker for 20 h. Purification of the mixture by HPLC did not yield ^{13}C labeled or unlabeled **1**.

2.2 Incorporation assays to further understand the in vitro LNKS activity for the production of the shunt metabolite pyrones

LovB should be able to transform **17** into the labeled pyrones **3a** and **4a**. It has been demonstrated previously by our group that **3** and **4** isolated from the culture medium of *A. nidulans lovB* are transformed to the more stable **18** and **19** by hydrogenation.[52] The pyrones **3a** and **4a**, if made, can be transformed to **18a** and **19a** (Scheme 5). Due to the small amounts of **3** and **4** isolated from fermentations of the *A. nidulans lovB* culture and their instability, the synthesis of **18** and **19** was undertaken to obtain a standard for dilution assays. The ^{13}C label of **18a** and **19a** could be detected by ^{13}C-NMR. Radiolabeled analogs of **18** and **19** will also be tested for using ^{14}C malonyl-CoA as a substrate.

Scheme 5: Proposed conversion of triketide **17** into the pyrones **3a** and **4a**

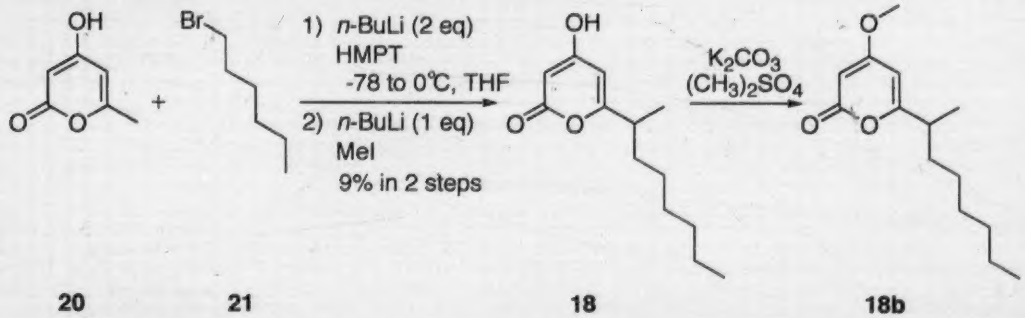


malonyl-CoA
+
Ac-CoA
or
17
LovB
short pyrone
3
labeled (*)
3a
long pyrone
4
labeled (*)
4a
H_2 Pd/C
EtoAc
short pyrone
18
labeled (*)
18a
long pyrone
19
labeled (*)
19a

2.2.1.1 Synthesis of the short saturated pyrone 18 by substitution

The synthesis of the short saturated pyrone **18** was straightforward. Alkylation of 4-hydroxy-6-methyl-2-pyrone (**20**) has been performed previously.[103] Using a modification of the procedure of Poulton and Cyr,[103] the commercial pyrone **20** was deprotonated with two equivalents of *n*-butyllithium. The first deprotonation occurs at the hydroxyl group and the second proceeds at the methyl group of C-1′. The pyrone is then C-alkylated with 1-bromohexane (**21**), followed by a third deprotonation at the methylene of C-1′ and alkylation with methyl iodide in the same pot to yield the short pyrone **18** (Scheme 6). The spectroscopic properties of the product match those reported by Dr. Karine Auclair.[52] Compound **18** can also be further methylated to give **18b**.

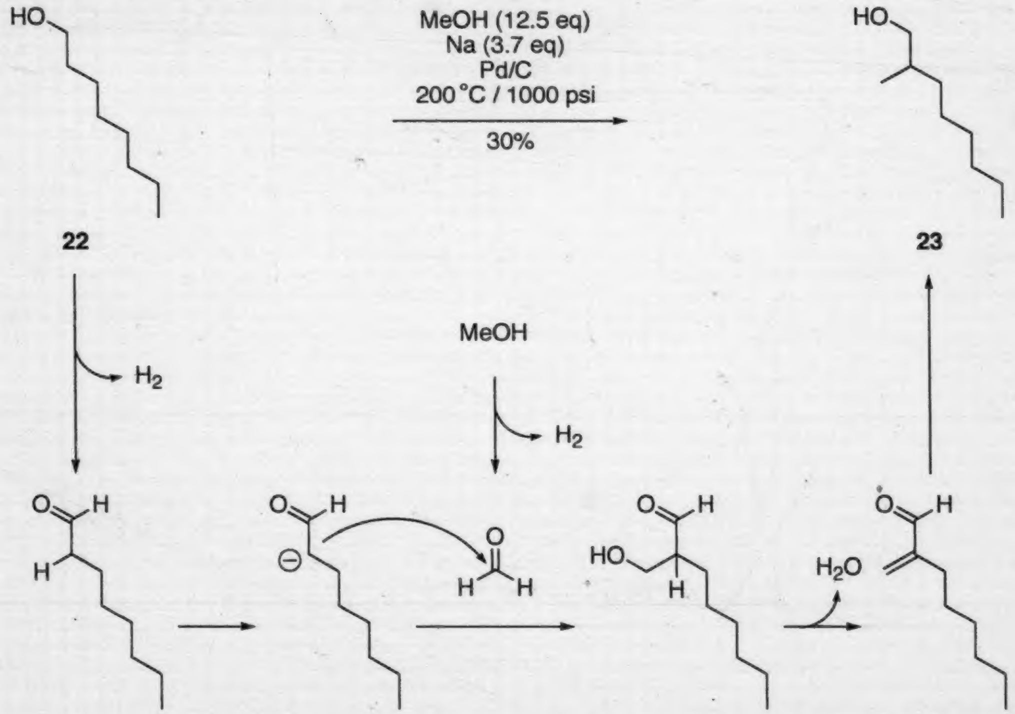
Scheme 6: Synthesis of the short saturated pyrone **18**


OH
Br
+
1) n-BuLi (2 eq)
HMPT
-78 to 0°C, THF
2) n-BuLi (1 eq)
MeI
9% in 2 steps
OH
K_2CO_3
$(CH_3)_2SO_4$
20
21
18
18b

2.2.1.2 Investigation of the synthesis of the long saturated pyrone 19 by substitution

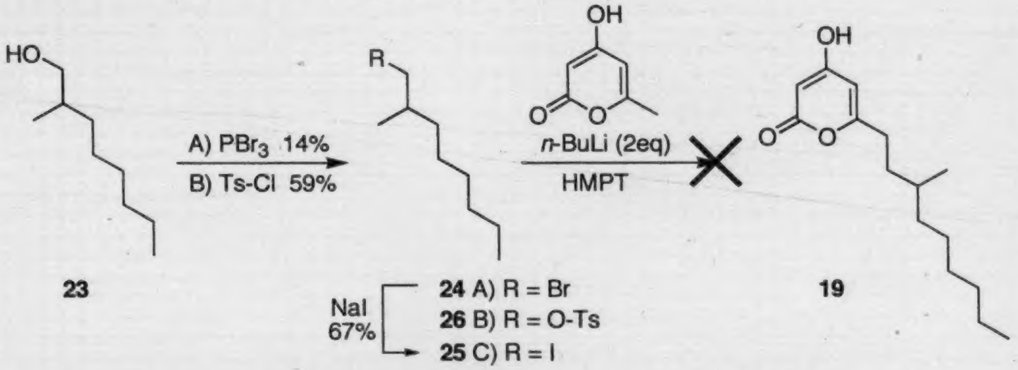
The synthesis of the long unsaturated pyrone **19** was attempted using a similar method as described for the preparation of **18**. The corresponding bromide was not commercially available and was thus synthesized from the appropriate alcohol **23**. The alcohol **23** was prepared *via* a Guerbet reaction using octanol (**22**) and methanol (Scheme 7).[104-106]

Scheme 7: Mechanism of formation of alcohol **23**


HO
22
MeOH (12.5 eq)
Na (3.7 eq)
Pd/C
200 °C / 1000 psi
30%
HO
23
H2
MeOH
H2
O
H
H
O
H
⊖
O
H
H
O
H
HO
H
H2O
O
H

The alcohol **23** was converted to the corresponding bromide **24**. The attempt to generate the long pyrone **19** with **24** using the same conditions as for the short pyrone **18** was unsuccessful. In order to enhance the electrophilicity of the reagent, the iodide **25** was generated from the bromide using the Finkelstein reaction.[107] The iodide **25** was also ineffective in generating the product **19**. The tosylate **26** of the alcohol **23** was also made,[108] but better results could not be obtained (Scheme 8).

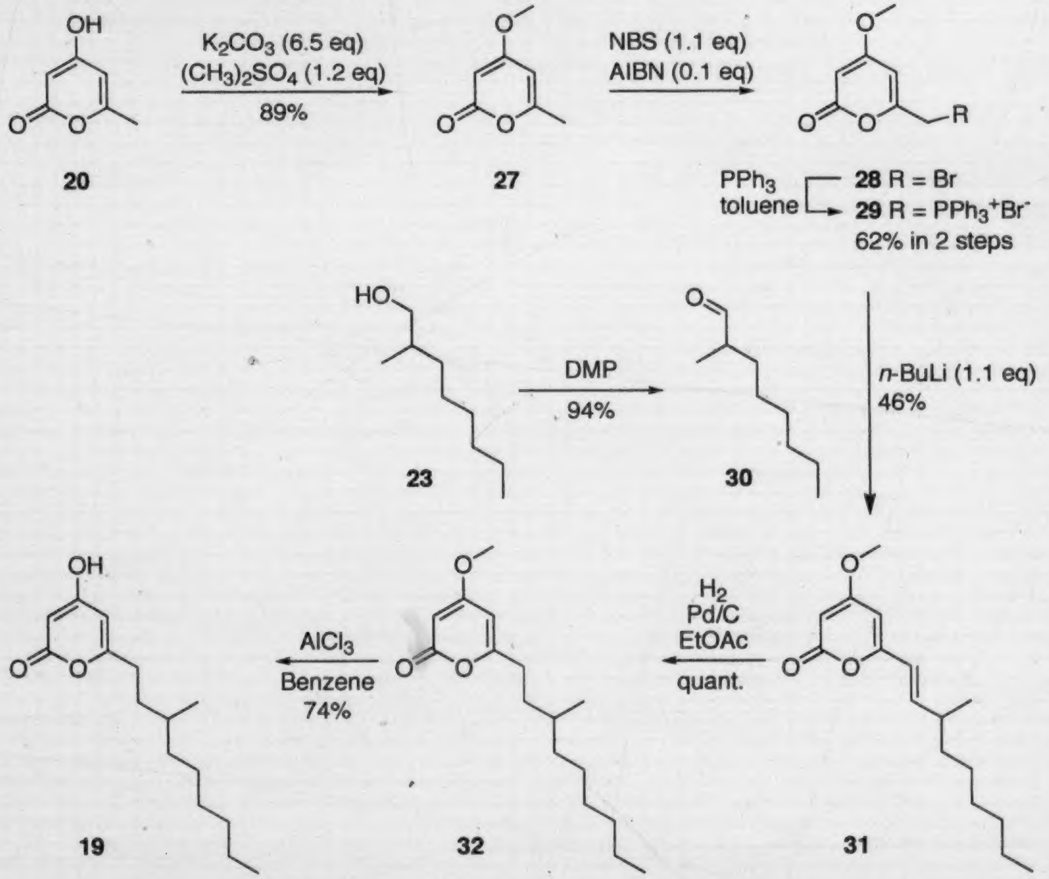
Scheme 8: Studies towards the synthesis of the long unsaturated pyrone **19**



2.2.1.3 Synthesis of the long saturated pyrone 19 by a Wittig reaction

Due to the lack of success generating **19** by substitution, another method was needed. With the knowledge that during standard hydrogenation, the pyrone ring is not reduced,[52] a Wittig reaction involving a pyrone ylid with the aldehyde generated from **23** followed by hydrogenation was attempted (Scheme 9). The salt **29** has been previously made.[109] Compound **20** was O-methylated to give **27**, and this was brominated on the 6-methyl group to yield **28**. Because only the desired brominated product will react with PPh_3, the reaction was carried forward without purification, to generate the salt **29**. The alcohol **23** was oxidized to the aldehyde **30** with Dess-Martin periodinane (DMP)[110-112] and the Wittig reaction was performed to yield the alkene **31**. The exocyclic double bond was hydrogenated and the resulting pyrone **32** was demethylated[113] with $AlCl_3$ to give the long saturated pyrone **19** (Scheme 9).

Scheme 9: Synthesis of the long saturated pyrone


K_2CO_3 (6.5 eq)
$(CH_3)_2SO_4$ (1.2 eq)
89%
20
27
NBS (1.1 eq)
AIBN (0.1 eq)
PPh3
toluene
28 R = Br
29 R = PPh3+Br-
62% in 2 steps
DMP
94%
23
30
n-BuLi (1.1 eq)
46%
H2
Pd/C
EtOAc
quant.
AlCl3
Benzene
74%
19
32
31

2.2.1.4 Studies on incorporation of the ^{13}C labeled triketide-NAC thioester 17 into the saturated pyrones 18 and 19 using cell free extracts of *A. nidulans lovB*

An active CFE of LovB was prepared. The labeled triketide **17** was incubated with the LovB CFE for 1.5 h before adding FAD, NADPH, SAM and malonyl-CoA and agitating the mixture on an orbital shaker for 8 h. The pyrones **18** and **19** were added to the mixture and the reaction quenched. The crude extract was subjected to hydrogenation. Purification of this mixture did not reveal a ^{13}C label in the pyrones.

It is possible, although highly unlikely, that the triketide is not along the biosynthetic pathway and thus does not get incorporated. LovB activity has been measured by the UV assay as described in the fermentation section. If the increase in absorbance at 360 nm is due to the production of the pyrones **3** and **4** from Ac-CoA and malonyl-CoA then it should be possible to detect these if ^{14}C malonyl-CoA is used as a substrate.

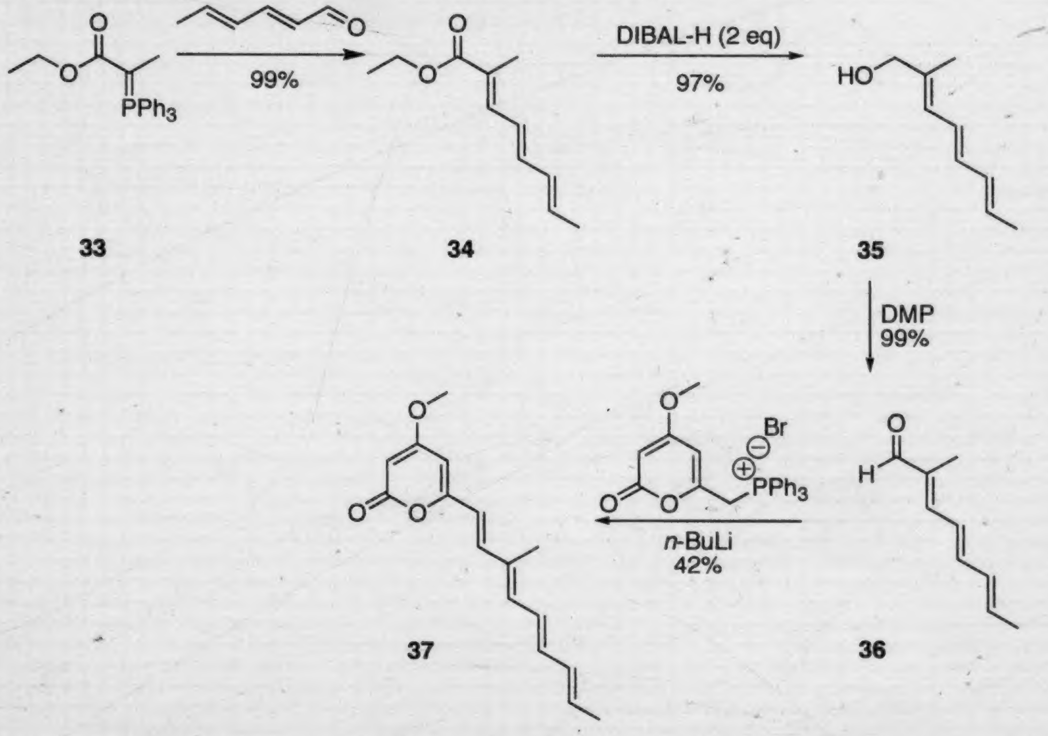
2.2.2.1 Studies on incorporation of ^{14}C labels into the saturated pyrones 18 and 19 using cell free extracts of *A. nidulans lovB*

An active LovB CFE was prepared. FAD, NADPH, SAM, Ac-CoA and malonyl-CoA (unlabeled and ^{14}C labeled) were added to the CFE. After 5.5 h, the reaction mixture was diluted with **18** and **19** and the mixture was hydrogenated. The crude mixture was purified by HPLC, but the re-isolated pyrones **18** and **19** were not radioactive. The majority of the radioactivity was in the aqueous extract. The radioactivity that was in the organic layer was due to malonic acid. Commercial malonic acid was acidified and extracted with EtOAc using the same procedure as above. Approximately 15% was extracted into the organic phase and had the same retention time on the HPLC as where the radioactivity appeared. It is likely that much of the radioactivity in the aqueous phase was also due to malonic acid and/or malonyl-CoA. A possible reason for the lack of incorporation of a radioactive label is that the process is too long (although the reaction time to purification was less than 24 h). Previous studies in our group have shown that the pyrones **3** and **4** are quickly degraded. It seemed that perhaps the extra step to hydrogenate the pyrones allows time for **3** and **4** to be degraded. In order to test this hypothesis the synthesis of the pyrones **3** and **4** was undertaken.

2.2.2.2 Synthesis of the methylated long unsaturated pyrone 37

As the Wittig salt of the pyrone **29** had already been synthesized, a method using this procedure to generate the long pyrone **4** was envisioned (Scheme 10). The synthesis of the appropriate unsaturated aldehyde **36** was initiated from sorbic aldehyde by treatment with the ylid **33** to generate the ethyl ester **34**. The ester was reduced to the alcohol **35** and then oxidized to aldehyde **36**. The Wittig olefination using the ylid derived from **29** generated the O-methylated pyrone **37** (Scheme 10).

Scheme 10: Synthesis of the methylated long pyrone **37**

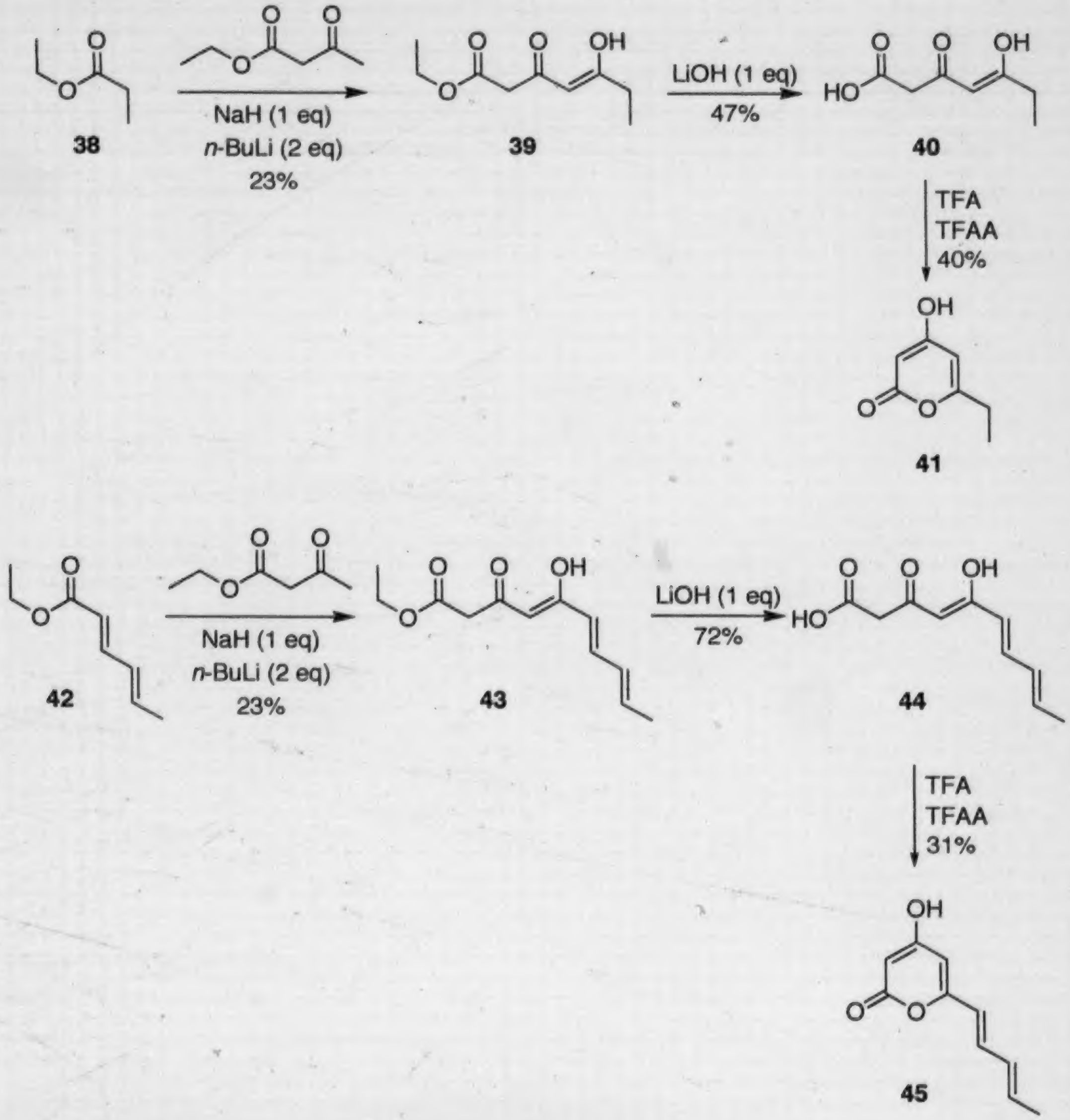


Demethylation of **37** to yield **4** proved to be difficult. AlCl$_3$, iodotrimethylsilane,[114] aqueous HBr with LiBr,[115] sodium thiobutoxide (modified from Salomon *et al*),[116] BBr$_3$,[117] and AlN$_3$[118] were unable to successfully remove the methyl group. The methylated pyrone **37** was either unreactive or decomposition occurred.

2.2.2.3 Trial biomimetic synthesis of pyrones (41 and 45)

There is precedent that 3,5-diketo acids can cyclize to form 4-hydroxy-2-pyrones.[119] If the substitution at the terminal end of the acid is appropriate, this method can in principle biomimetically generate the pyrones **3** and **4**. Two sets of trial reactions (saturated and unsaturated side chains) were done to test this method. The β-keto esters **39** and **43** were synthesized from ethyl acetoacetate and the appropriate ester using the methods of Weiler.[120] The esters **39** and **43** were then hydrolyzed with LiOH and the acids **40** and **44** were cyclized[119] to the corresponding pyrones **41** and **45** respectively (Scheme 11).

Scheme 11: Trial biomimetic synthesis of pyrones

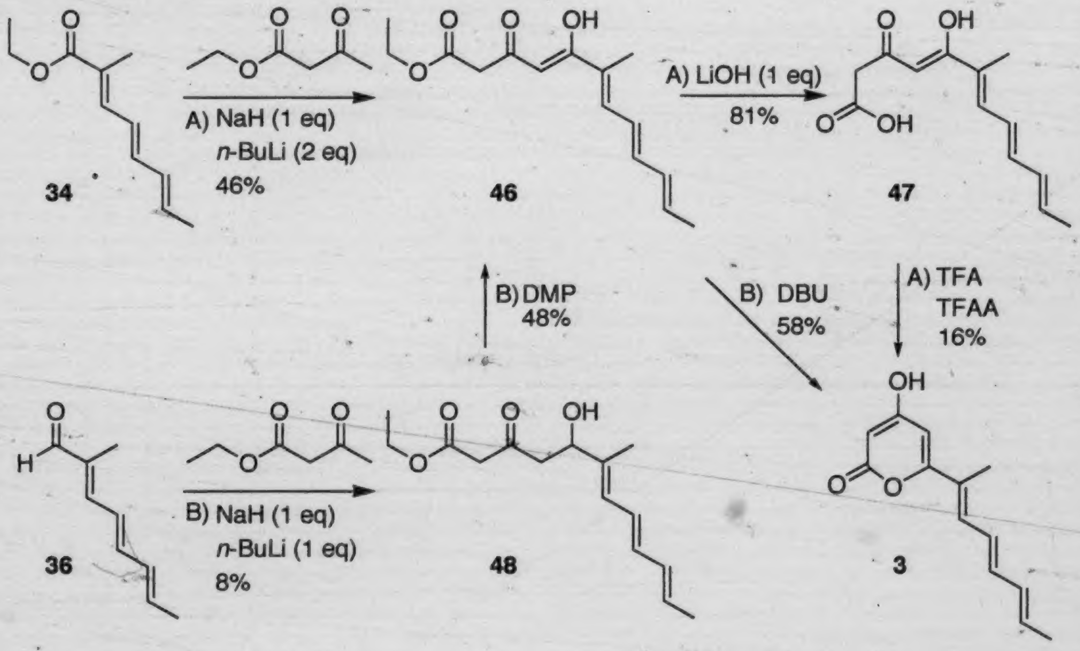

38
NaH (1 eq)
n-BuLi (2 eq)
23%
39
LiOH (1 eq)
47%
40
TFA
TFAA
40%
41
42
NaH (1 eq)
n-BuLi (2 eq)
23%
43
LiOH (1 eq)
72%
44
TFA
TFAA
31%
45

2.2.2.4 Synthesis of the short unsaturated pyrone 3

The short pyrone **3** was synthesized using the same method used for **41** and **45**. The ester **34** was condensed with ethyl acetoacetate to yield the β-keto ester **46** (Scheme 12). This was a poor yielding reaction due to many side products being formed with very similar chromatographic properties. The β-keto ester **46** was then hydrolyzed to the acid

47 and cyclized to yield the desired short pyrone **3** (Scheme 12) with the same spectroscopic properties as the natural product isolated by Dr. Karine Auclair.[52] An alternative route to **3** was realized following recent work by Trauner.[121] The β-keto ester **46** was prepared through an aldol condensation of ethyl acetoacetate with the aldehyde **36** to yield alcohol **48**. Compound **48** was oxidized with DMP to give the desired product **46**. The β-keto ester was cyclized directly to the pyrone **3** using the base DBU (Scheme 12). Overall, the second method was lower yielding and contained more steps to form the aldehyde compared to first approach. However, a combination of the two methods using the coupling reaction of the first method with the cyclization of second approach gives the desired product in three steps from commercial starting materials.

Scheme 12: Synthesis of the short pyrone **3**



2.2.2.5 Studies on incorporation of ^{14}C labels into the unsaturated pyrone 3 using cell free extracts of *A. nidulans lovB*

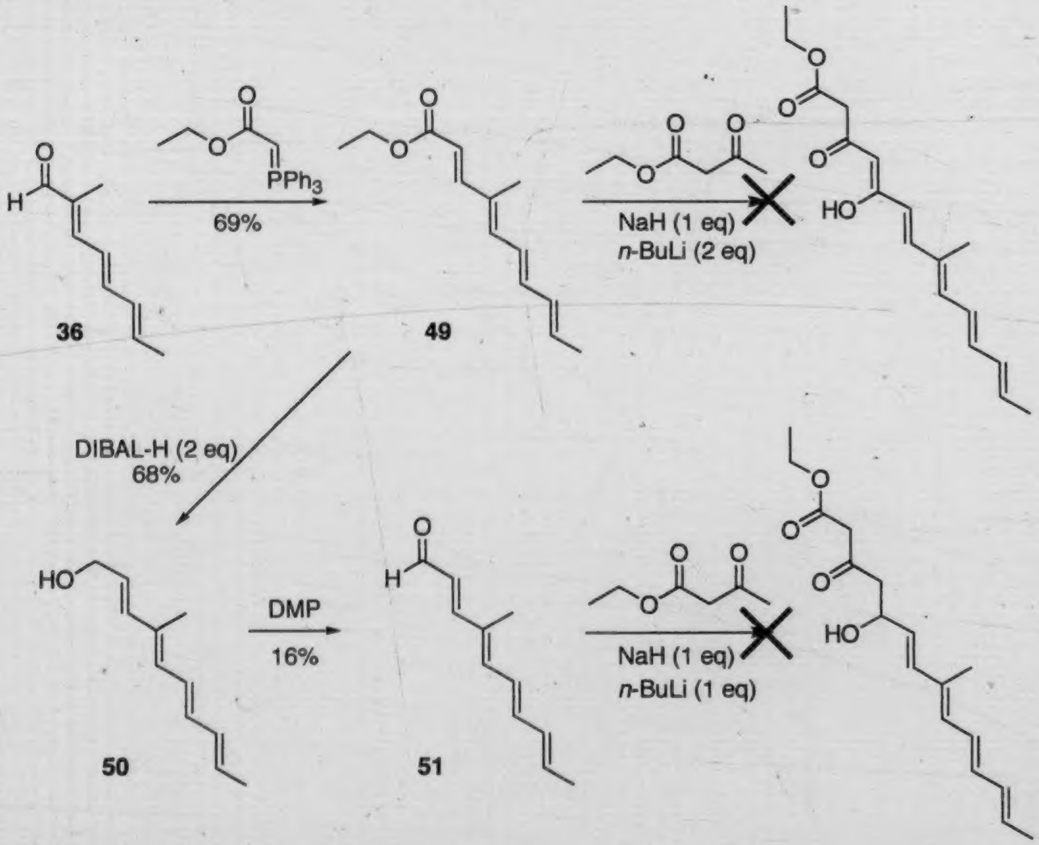
With the short pyrone **3** available, a CFE of LovB was prepared. FAD, NADPH, SAM, Ac-CoA and malonyl-CoA (both ^{14}C labeled and unlabeled) were added and the reaction mixture was shaken for 20 h, at which time a small amount of **3** was added. The reaction mixture was purified by HPLC, however the peak corresponding to the reisolated **3** was not radioactive. Experiments run in parallel with broken cells, boiled broken cells and an ultrafiltered fraction yielded the same results as with the CFE. The UV activity test of this LovB batch seemed to indicate that the activity was not reproducible.

As the possiblity exists that the spores died over time or had lost the *lovB* gene, the original culture spores were grown and an active CFE extract of LovB from this new batch was prepared. The activity as determined by the UV assay for pyrones was again reproducible. Part of this CFE was boiled for 30 min and used as a blank. To the blank and CFE was added FAD, NADPH, SAM, Ac-CoA and malonyl-CoA (both ^{14}C labeled and unlabeled). The reaction mixture was shaken for 20 h before addition of the short pyrone **3** and purification by HPLC. The peak corresponding to the short pyrone **3** was again not radioactive. The majority of the radioactivity eluted with the same retention time as malonic acid and malonyl-CoA. To confirm that these peaks were not the open acid form of the pyrone, and to exclude the possibility that the pyrone did not cyclize, the open acid **47** was injected. The retention time of open acid **47** did not correspond to the early radioactive peaks. The UV activity assay indicated that the peak absorbance occurred after 9 h with a decrease in absorbance being seen there after, possibly indicating the pyrones are being degraded. As the assay was run for 20 h, perhaps the pyrones degraded too quickly. The last reaction was repeated with termination of the reaction after 9 h. Purification of this reaction mixture by HPLC also did not yield radioactive pyrone.

2.2.2.6 Investigations towards the biomimetic synthesis of the long unsaturated pyrone 4 with ethyl acetoacetate

To synthesize the long pyrone **4** a similar approach to that used for the generation of **3** was undertaken. The necessary ester **49** was prepared from the aldehyde **36** with the appropriate ylid. Numerous attempts to couple **49** with ethyl acetoacetate were unsuccessful, and more than 90% of the starting material was recovered (Scheme 13). The ester **49** was reduced to the alcohol **50**, which was oxidized to the aldehyde **51**. An aldol coupling with ethyl acetoacetate following the Trauner procedure[121] did not yield the desired alcohol (Scheme 13).

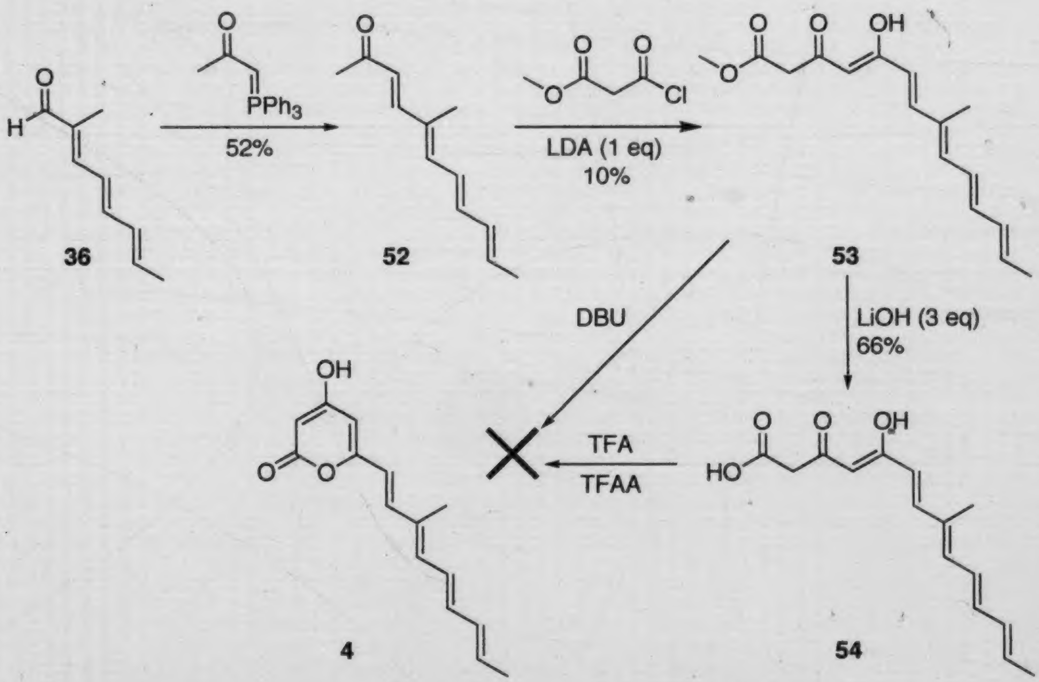
Scheme 13: Studies towards the synthesis of the long pyrone **4**



2.2.2.7 Investigations towards the biomimetic synthesis of the long unsaturated pyrone 4 with acetoxyacetyl chloride

Another way to potentially generate the β-keto ester of the tetraene is to have the tetraene portion be the nucleophile instead of the electrophile. To this end the tetraene methyl ketone **52** was generated from the aldehyde **36** through a Wittig reaction. The methyl β-keto ester **53** of the tetraene was successfully made through reaction of the ketone **52** and acetoxyacetyl chloride. The direct cyclization using DBU did not yield the desired product. The methyl ester was cleaved to the acid **54** and cyclization was attempted, but again no desired product was obtained (Scheme 14).

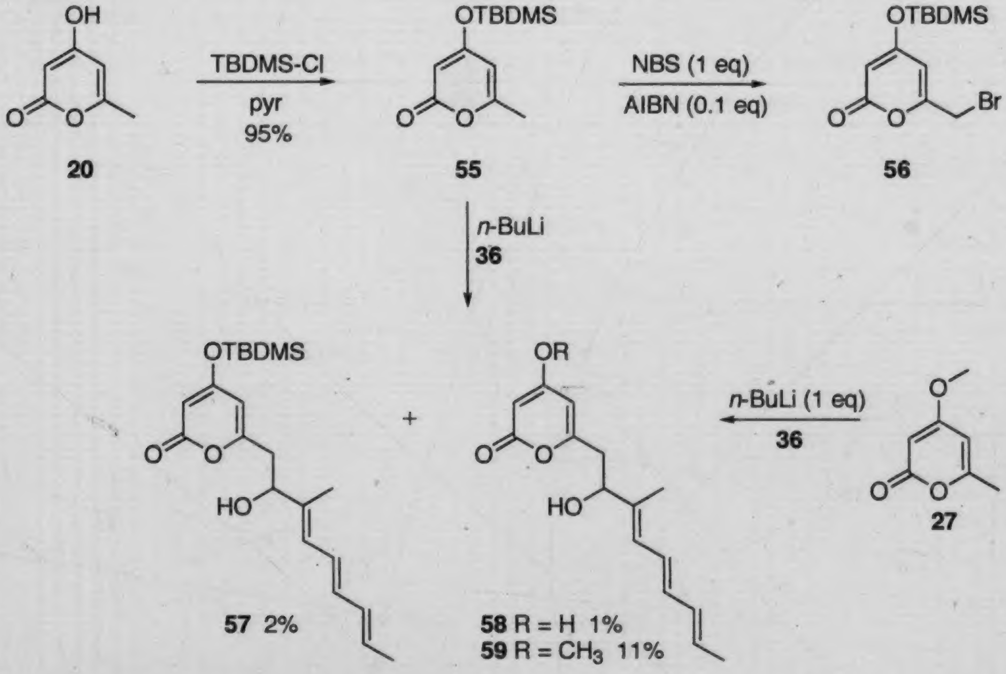
Scheme 14: Studies towards the synthesis of the long pyrone **4**



2.2.3.1 Synthesis of silyl-protected pyrone 55 and chain elongation

The previous results suggested that a new method would be required to make the long pyrone **4**. A method similar to that shown in (Scheme 10) with a silyl protecting group for the 4-hydroxy group instead of a methyl should allow for the same coupling step and easier removal of the protecting group to yield the long pyrone **4**. The pyrone **20** was protected as its *t*-butyl dimethyl silyl ether **55**. The bromide **56** was prepared and reacted further in an attempt to make the Wittig salt (Scheme 15). The purified salt could not be obtained and the semi-purified material was unable to undergo a successful Wittig reaction with **36**. During the synthesis of prosolanopyrone II,[122] a related pyrone is able to undergo an aldol reaction at the 6-methyl group with an aldehyde, followed by tosylation and elimination of the alcohol to yield a new alkene. A similar approach was attempted with the silyl-protected pyrone **55**. Compound **55** was treated with *n*-BuLi followed but the addition of the aldehyde **36**. A very small amount of product **57** was isolated. The deprotected product **58** was also isolated in low yield (Scheme 15). Attempts to tosylate and eliminate the alcohols **57** and **58** did not provide the desired products. The methyl-protected pyrone **27** also underwent an aldol reaction with **36** to yield **59**. However, an attempt to demethylate **59** with $AlCl_3$ was unsuccessful. Due to the low yield of these reactions, this scheme was not pursued further.

Scheme 15: Synthesis of silyl-protected pyrone **55** and its aldol reaction with **36**



2.2.3.2 Synthesis of protected pyrones

To protect the alcohol **20**, groups more readily removed than methyl and more stable to bromination/Wittig salt formation than silyl were considered. Compound **20** was successfully protected with trityl to give **60** and methoxymethanol to generate **61**. However, during the attempted protection as THP ether, none of the desired product was obtained. Instead the protecting group was attached at the 3 position of the ring to give

64. This was confirmed with a crystal structure of **64** (Figure 17). The MOM protected pyrone **61** was converted to the bromide **62**. The formation of the Wittig salt proceeded as desired and was detected by low-resolution electrospray mass spectrometry (LRMS-ES), however complete purification was not achieved (Scheme 16).

Scheme 16: Protection studies on pyrone **20**

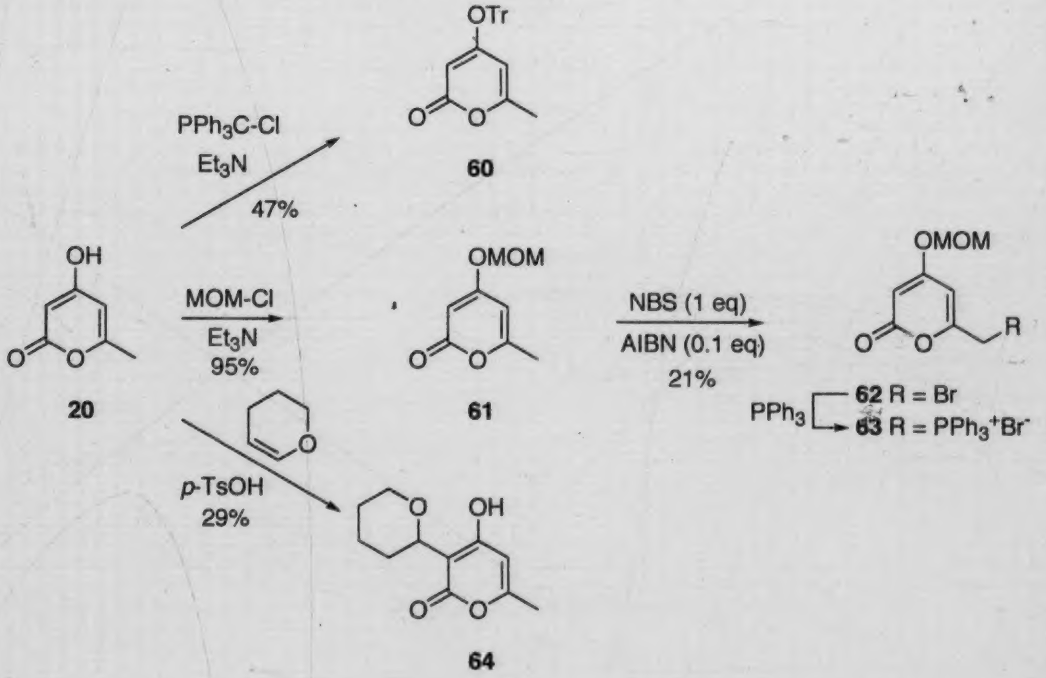
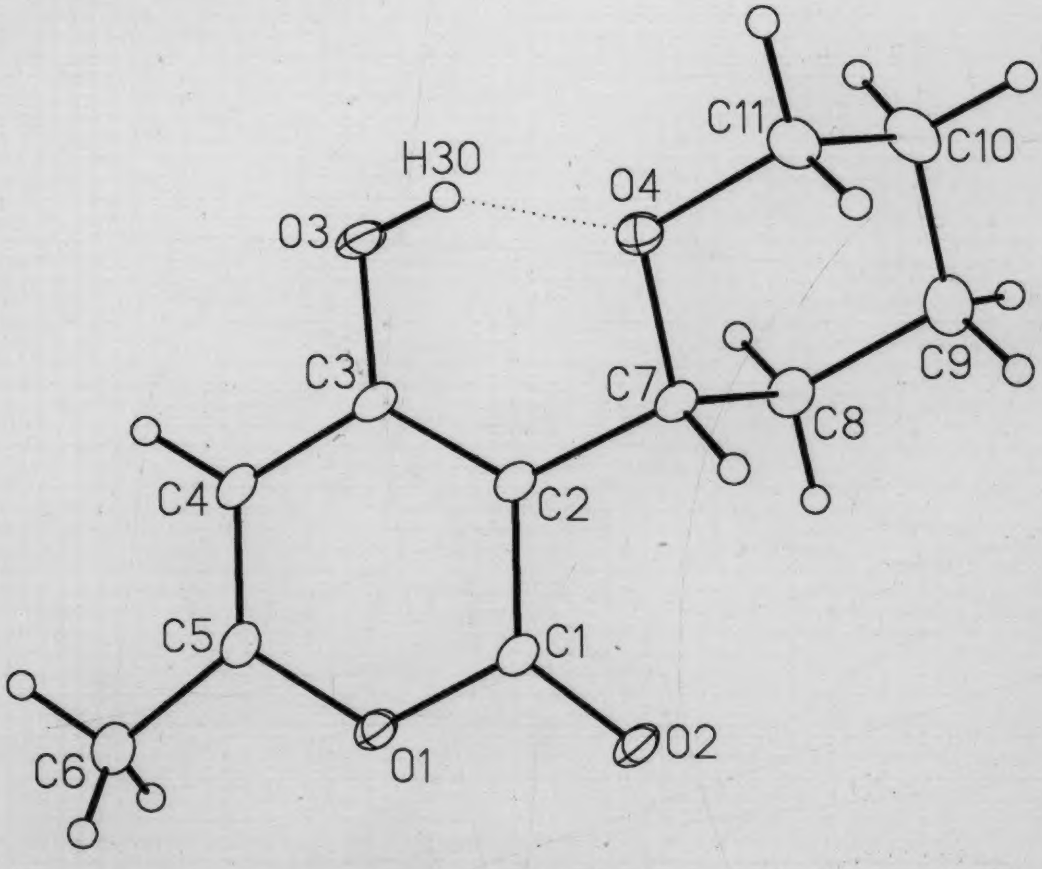

OTr
PPh3C-Cl
Et3N
47%
60
OH
20
MOM-Cl
Et3N
95%
OMOM
61
p-TsOH
29%
OH
64
NBS (1 eq)
AIBN (0.1 eq)
21%
OMOM
R
PPh3
62 R = Br
63 R = PPh3+Br-

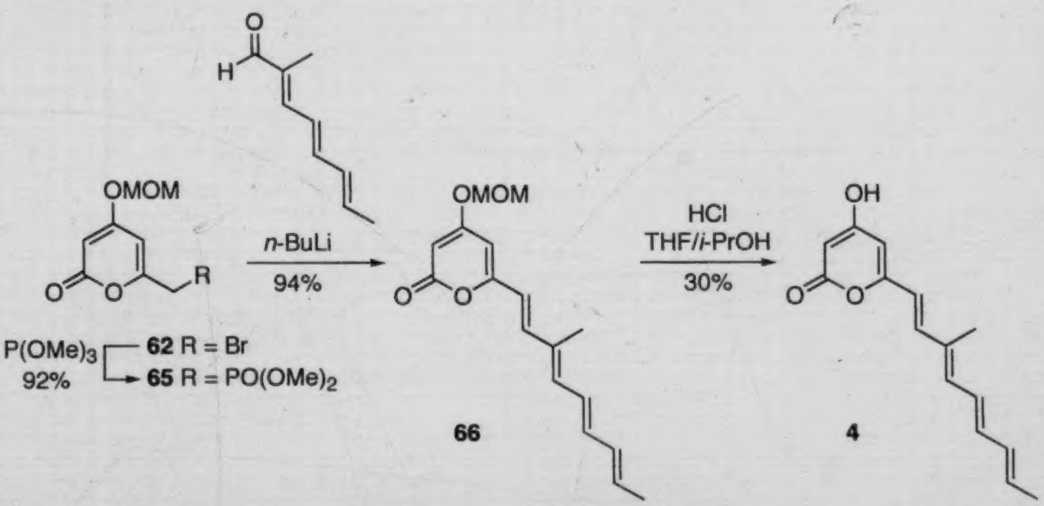
Figure 17: Crystal structure of **64**



2.2.3.3 Synthesis of the long unsaturated pyrone 4

Due to the difficulty in purification of the Wittig salt **63** by recrystallization, it was hypothesized that using a phosphonate that can be purified by column chromatography could be a better alternative. This was undertaken and performed by Ms. Brandy Chen in the our group. Once the phosphonate was prepared, the olefination with **36** proceeded as expected, followed by deprotection of the MOM group with HCl to yield the desired pyrone **4** (Scheme 17).

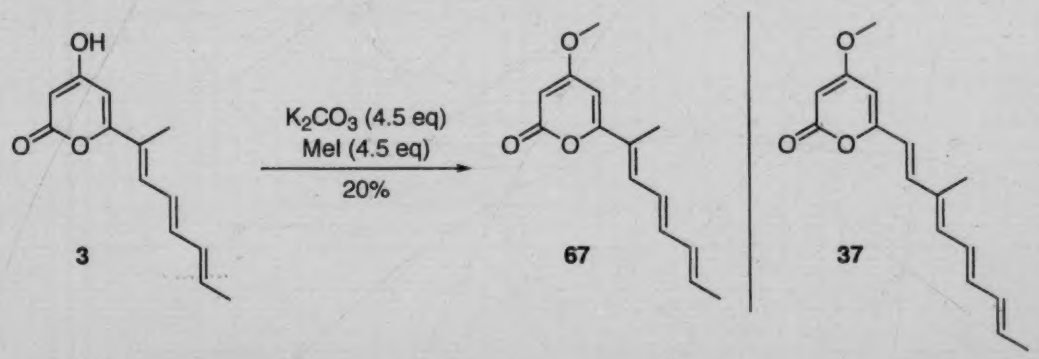
Scheme 17: Synthesis of the long pyrone **4** by Ms. Brandy Chen


OMOM
R
P(OMe)3
92%
62 R = Br
65 R = PO(OMe)2
n-BuLi
94%
OMOM
66
HCl
THF/i-PrOH
30%
OH
4

2.2.3.4 Synthesis of the short methylated unsaturated pyrone 67

As it is possible that the isolated pyrones are more stable if protected as their methyl ethers, the short pyrone **3** was methylated to yield **67** (Scheme 18) to use as a standard along with the long methylated pyrone **37**.

Scheme 18: Synthesis of the short methylated pyrone **67** and structure of **37**


OH
3
K2CO3 (4.5 eq)
MeI (4.5 eq)
20%
67
37

2.2.4.1 Studies on isolation of pyrones from fermentation of *A. nidulans lovB* as their methyl ethers 37 and 67

A culture of *A. nidulans lovB* was grown and NaOAc was added as a carbon source to increase the production of the pyrones. Once the fermentation was complete, the culture broth was extracted and submitted to methylation conditions with potassium carbonate and iodomethane. HPLC of the crude mixture did not provide methylated pyrones.

2.3 Studies on in vitro production of dihydromonacolin L (1) by A. nidulans lovB+C

2.3.1 Studies on incorporation of ^{14}C labels into dihydromonacolin L (1) using cell free extracts of *A. nidulans lovB+C*

An active CFE of LovB+C was prepared *A. nidulans lov*B+C as described in the experimental section. This CFE was incubated on an orbital shaker with FAD, NADPH, SAM, Ac-CoA and malonyl-CoA (^{14}C labeled and unlabeled) for 17 h. The mixture was extracted with CH_2Cl_2. The crude mixture was purified by preparative TLC, but no radioactivity was isolated in the spot corresponding to **1**. In a reaction similar to the one just described, extraction of the reaction mixture with EtOAc followed by lactonization by refluxing in toluene with a CaH_2 soxhlet and purification did not yield radioactive **1**.

2.3.2 Studies on incorporation of ^{14}C labels into dihydromonacolin L (1) using cell free extracts of *A. nidulans lovB+C* using lower centrifugation speeds

It is possible that during the preparation of the CFE the LovB and LovC enzymes are separated from each other, even though previous research by Hutchinson and Kennedy and our group indicate that the two proteins associate strongly. This is based on studies in which the His-tagged LovC and LNKS co-purify (unpublished data). To avoid possible separation problems due to centrifugation, the ground LovB+C mycelia was centrifuged at a lower speed (1000 and 3000 compared to 8000 rpm). Performing the same reaction as above with extraction into EtOAc and lactonization, followed by purification by preparative-TLC and HPLC did not yield radioactive dihydromonacolin L (**1**).

2.3.3 Studies on incorporation of ^{14}C labels into dihydromonacolin L (1) using crude broken cells of *A. nidulans lovB+C*

To test if another required element is missing from the CFE, crude broken cells were examined. The mycelia were filtered and frozen immediately in liquid N_2 and ground into a fine powder with a mortar and pestle. The powder was suspended in extraction buffer and FAD, NADPH, SAM, Ac-CoA and malonyl-CoA (^{14}C labeled and unlabeled). After 17 h, the reaction mixture was extracted with EtOAc and lactonized. Purification of this mixture by preparative TLC and HPLC did not reveal radioactive dihydromonacolin L (**1**).

2.3.4 Studies on incorporation of ^{14}C labels into dihydromonacolin L (1) using cell free extracts of *A. nidulans lovB+C* in the presence of ATP

Studies performed by Reeves *et al* indicate that ATP is required for malonyl-CoA to be loaded *in vitro*.[53] To this end a CFE of LovB+C was prepared and was incubated with ATP, FAD, NADPH, Ac-CoA, and malonyl-CoA (^{14}C labeled and unlabeled) for 17 h. The mixture was extracted with EtOAc and CH_2Cl_2. However, neither of these fractions contained radioactive **1** by HPLC.

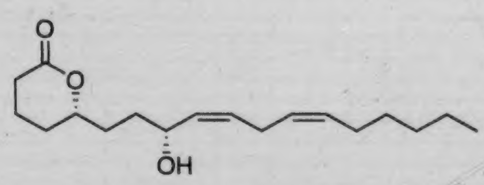
2.3.5 Isolation of the sex factor psiAα 68

In order to see whether the LovB and the LovC proteins can be reunited in an active form after growth in separate cells, a CFE of *A. nidulans lovB* was prepared and combined with a CFE of an *A. nidulans lovC* strain. This CFE was incubated with FAD, NADPH, SAM, Ac-CoA and malonyl-CoA for 40 h. The mixture was extracted with

CH_2Cl_2 and lactonized by heating. Purification of this mixture by HPLC yielded a peak corresponding to the retention time and R_f of dihydromonacolin L (**1**). However, ^{1}H-NMR and high resolution mass spectrometry (both EI and ES) indicated that this compound was not **1**. Mass spectrometry indicated the compound contained one carbon less than **1**. A literature search revealed that the sex factor psiAα[123] **68** (Figure 18) had the correct MW and the ^{1}H-NMR spectra matched that of the isolated compound. The sex factor **68** was also isolated from a similar experiment as the one just described except the CFEs were both ultrafiltered prior to the reaction.

As labeling studies were not performed, it is unknown if **68** is just an isolate from the fermentation or whether it is produced *in vitro* by the necessary enzymes. A blank assay run excluding Ac-CoA and malonyl-CoA did not seem to produce **68**, although the amount of isolated compound was very small and did not yield a conclusive ^{1}H-NMR spectrum. Future studies would need to be performed to determine if **68** is produced *in vitro*.

Figure 18: *A. nidulans* sex factor psiAα **68**



68

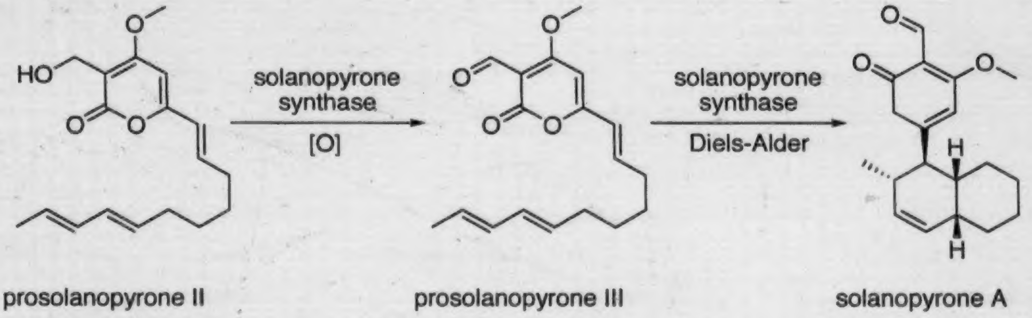
2.3.6 Significance

Studies performed at various stages of the enzyme purification in an attempt to incorporate advanced intermediates, or to obtain functioning *in vitro* enzymes indicate that the enzymes are not functioning correctly outside of the cells. With regard to the incorporation assays, it is possible that the active site (ACP) of the enzyme is essentially buried and thus shut off to substrates other than malonyl-CoA. It is also possible that during the process of generating the CFE or breaking the cells a crucial factor is lost that is necessary for the correct functioning of LovB as the short pyrone was not generated *in vitro* using CFE or broken cells. Another possibility for the pyrones not being made *in vitro* could be that they are not released by LovB and remain bound. It may be possible to test this hypothesis by treatment with a base to hydrolyze the pyrone from the enzyme after the necessary reaction has occurred. The C terminal domain of LovB has similarities to NRPS,[49] although the function of the C terminal domain remains unclear in LNKS and may be linked to some of its unusual properties.

2.4 Investigation towards prosolanopyrone II synthesis

Prosolanopyrone II is a natural product known to undergo an enzymatic Diels-Alder reaction after oxidation to the aldehyde (Figure 19). Prosolanopyrone II has previously been synthesized.[122] An alternative synthesis seemed possible based on our pyrone studies and was thus undertaken. Once the alcohol is produced, it would be possible to test oxidizing enzymes other than solanopyrone synthase to oxidize the product. The Diels-Alder reaction is reported to be very rapid once the aldehyde is formed.[122] If the oxidizing enzyme can generate the aldehyde, it may be possible that the Diels-Alder reaction can be guided by the active site to yield an enantiomerically pure product. This part of the project is being investigated by Mr. Jamie Côté in our group.

Figure 19: Solanopyrone biosynthesis


HO
O
O
O
solanopyrone synthase
[O]
O
O
O
O
solanopyrone synthase
Diels-Alder
O
O
O
H
H
prosolanopyrone II
prosolanopyrone III
solanopyrone A

With the Wittig salt **29** available an olefination with the appropriate aldehyde could yield the side chain. Installation of the CH_2OH group at C-3 is envisioned using formaldehyde or using dichloromethyl methyl ether (DCME) followed by reduction with $NaBH_4$ as previously reported[122] and shown in the trial reactions (Scheme 19). The necessary (6*E*,8*E*)-deca-6,8-dienal shown in (Scheme 20) was synthesized and generously donated by Mr. Jamie Côté.

2.4.1 Trial alkylation reaction of C-3 of pyrone 27

Trial reactions to insert the CH_2OH group on C-3 were attempted with the methyl protected pyrone **27** (Scheme 19). Treatment of **27** with DCME can effectively insert an aldehyde at C-3 to give **69**. If the reduction reaction was done without purification of **69**, no desired product was seen. Instead the dimethyl acetal protected aldehyde **71** was obtained. Purified **69** could be effectively reduced to the alcohol **70**. The alcohol **70** was also obtained directly from **27** by treatment with gaseous formaldehyde or *para*-formaldehyde with $TiCl_4$. The long chain pyrone **32** can also undergo the alkylation reaction at C-3 with DCME with either $TiCl_4$ or $SnCl_4$ to yield the aldehyde **72** (Scheme19).

Scheme 19: Trial alkyl addition to C-3 of pyrone **27** and **32**

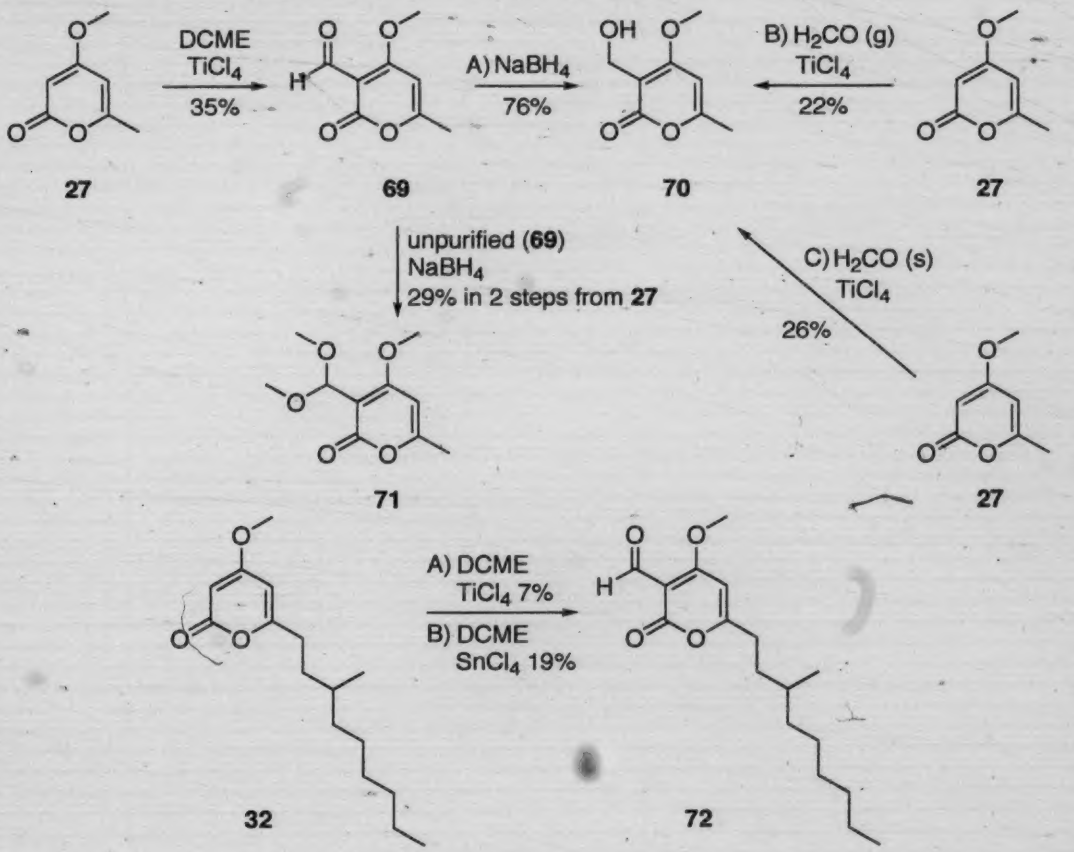
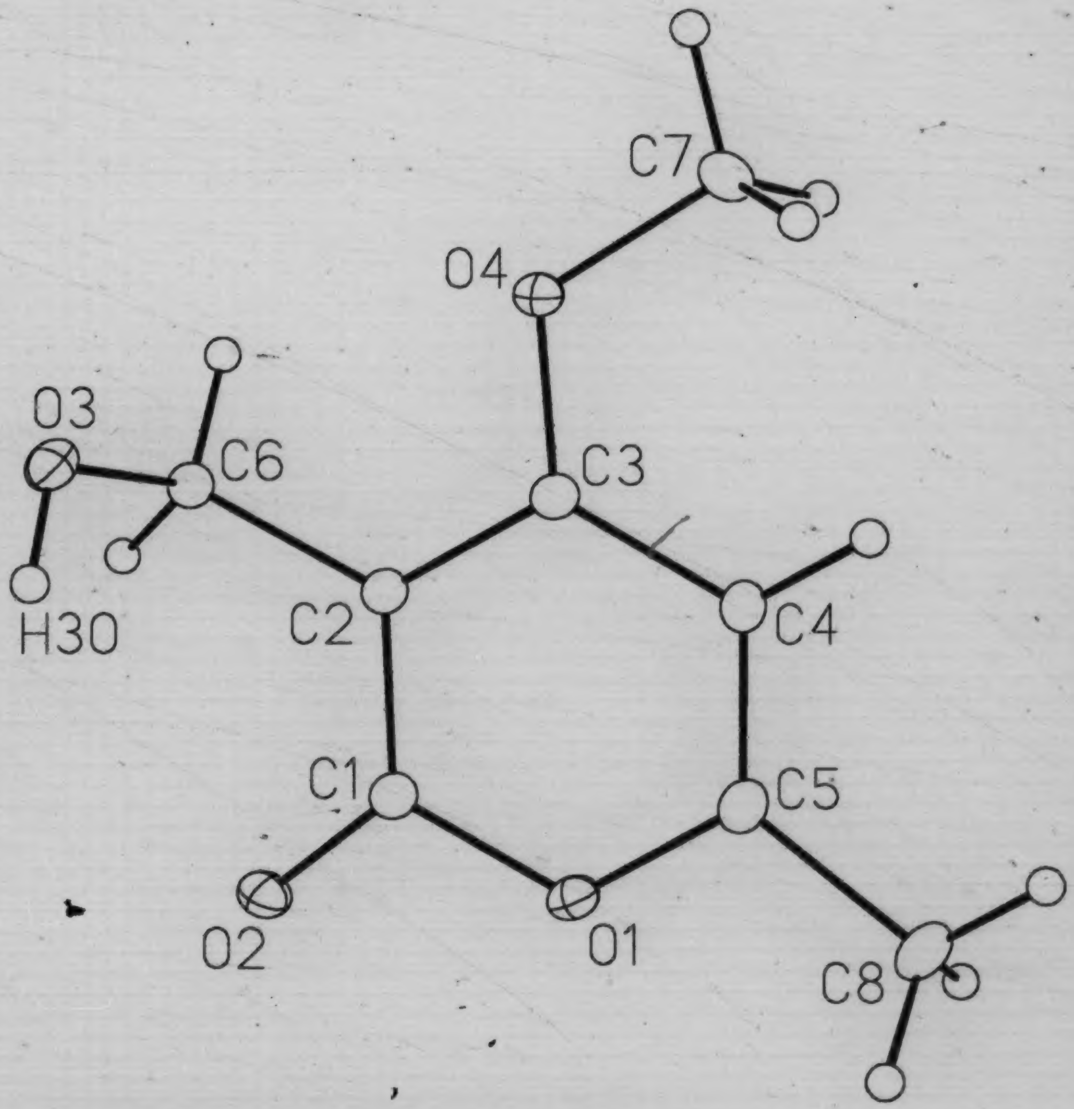

27
DCME
TiCl4
35%
69
A) NaBH4
76%
70
B) H2CO (g)
TiCl4
22%
27
unpurified (69)
NaBH4
29% in 2 steps from 27
C) H2CO (s)
TiCl4
26%
71
27
A) DCME
TiCl4 7%
B) DCME
SnCl4 19%
32
72

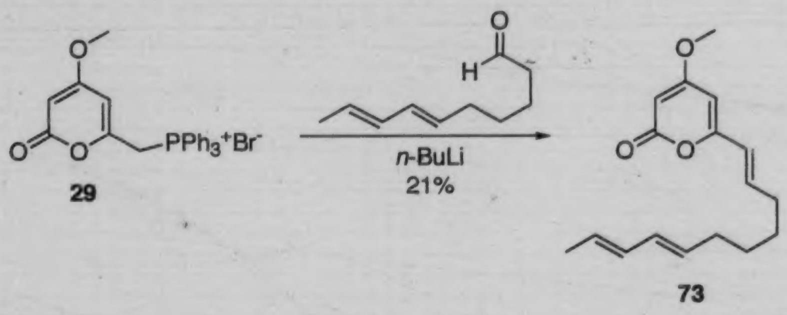
Figure 20: Crystal structure of **70**



2.4.2 Studies towards the synthesis of prosolanopyrone II

With a method available to alkylate at C-3 of the pyrones, synthesis of prosolanopyrone II was attempted. Compound **29** underwent a Wittig reaction with (6*E*,8*E*)-deca-6,8-dienal provided by Mr. Jamie Côté to yield the olefin **73** (Scheme 20). Unfortunately, numerous attempts to alkylate **73** at C-3 failed to provide the desired prosolanopyrone II. It is likely that the conditions required destroy the diene system of **73**.

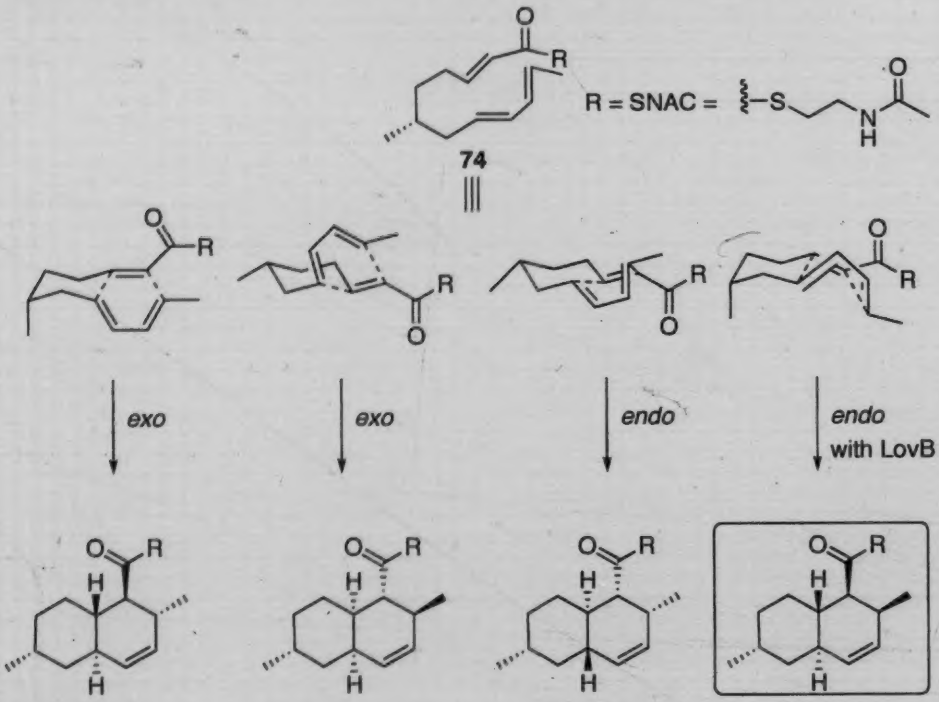
Scheme 20: Studies towards the synthesis of prosolanopyrone II



2.5 Investigation of the mechanism of the LNKS Diels-Alderase

LNKS has been shown to catalyze a Diels-Alder reaction.[77] *In vitro* experiments have shown that the presence of LNKS was crucial in the formation of the correct natural product, diastereomer **77**, during the cyclization of a hexaketide triene NAC ester **74**. If LovB was not present, only the diastereomers **75** and **76** were isolated (Scheme 21).[73,77]

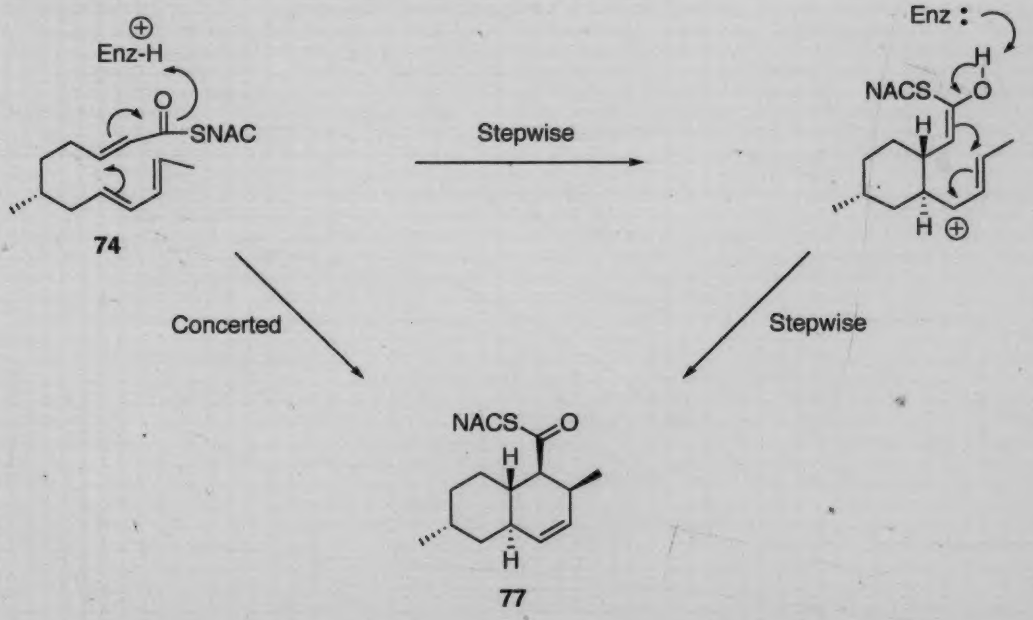
Scheme 21: Spontaneous and LovB catalyzed Diels-Alder reaction of **74**[77]


R = SNAC =
74
exo
exo
endo
endo
with LovB
O
R
H
N

	75		**76**		**77**
Spontaneous	1	:	1		
LovB	15	:	15	:	1

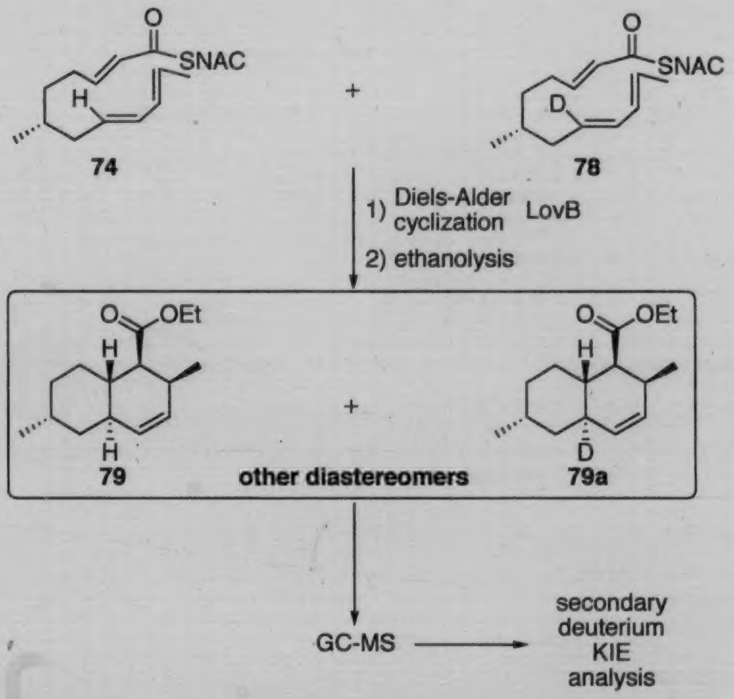
It remains unknown if the cyclization of **74** to **77** by LovB is indeed a true Diels-Alder reaction as proposed or if the reaction proceeds *via* a stepwise Michael-Aldol mechanism (Scheme 22).

Scheme 22: Proposed mechanisms of cyclization of **74**



Differentiation between a stepwise and concerted mechanism for possible Diels-Alder cyclization reactions has been studied extensively.[85-93] The two mechanisms can potentially be distinguished through theoretical calculations or experimentally by determining secondary deuterium kinetic isotope effects (KIEs).[85-93] The latter was pursued through the synthesis of various mono-deuterium labeled isotopomers of **74** (Figure 21), followed by a competitive reaction with unlabeled **74**. The products of the reaction will be a diastereomeric mixture of labeled and unlabeled NAC esters (**75**, **76** and **77**). The NAC esters are known to be difficult to separate and thus transformation by ethanolysis to their ethyl esters (no epimerization of the stereocenter occurs) allows for easier separation.[73] Only a small amount of the desired product **77** is formed enzymatically, making analysis difficult. By transformation of **77** to the ethyl ester **79** the volatility of the product will increase compared to the NAC ester **77** and analysis by gas chromatography mass spectrometry (GC-MS) should be possible. By using GC-MS the peak corresponding to each product should contain a mixture of the deuterated and unlabeled compound such as **79** and **79a**. The relative amounts of the two compounds can be compared by mass spectrometry and thus the secondary deuterium KIE calculated (Scheme 23).

Scheme 23: Proposed method for determination of the secondary deuterium KIE

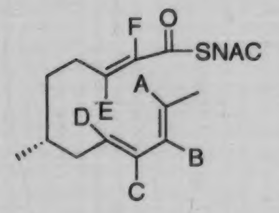


O
SNAC
H
74
+
O
SNAC
D
78
1) Diels-Alder cyclization
LovB
2) ethanolysis
O
OEt
H
H
79
+
other diastereomers
O
OEt
H
D
79a
GC-MS
secondary deuterium KIE analysis

2.5.1 Synthetic targets of the deuterated triene hexaketide-NAC thioester

The synthesis of **74** has previously been reported.[73] Using a modification of this method, the synthesis of the deuterated analogs was undertaken (Figure 21).

Figure 21: Proposed deuterated synthetic targets and expected isotope effects



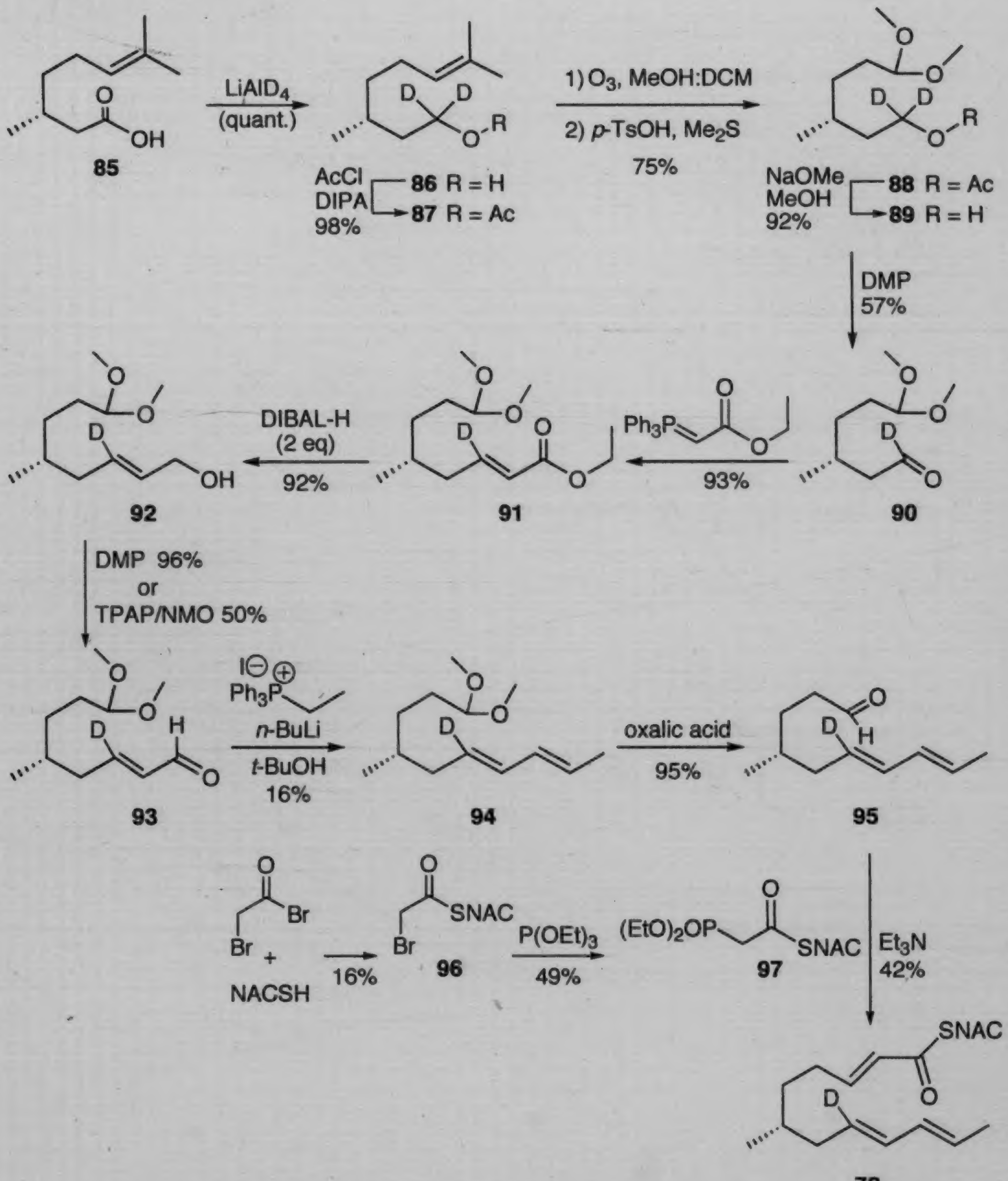
	A	B	C	D	E	F
80	D	H	H	H	H	H
81	H	D	H	H	H	H
82	H	H	D	H	H	H
78	H	H	H	D	H	H
83	H	H	H	H	D	H
84	H	H	H	H	H	D

Position	Concerted (expected)	Stepwise (expected)
A	< 1	~ 1
B	~ 1	> 1
C	~ 1	~ 1
D	< 1	~
E	< 1	< 1
F	< 1	~ 1

2.5.2 Synthesis of deuterated analog 78

The synthesis of **78** was performed using a modification of the Witter synthesis (Scheme 24).[73,124] The deuterium is incorporated during the first step by reduction of *R*-(+)-citronellic acid (**85**) with $LiAlD_4$ to generate the deuterated citronellol **86**,[125] which is then acylated to **87**. The acetate is then ozonized with reductive quenching in methanol to generate the dimethoxy protected aldehyde **88**. The acetate **88** is hydrolyzed to the alcohol **89**, which is oxidized to the aldehyde **90**. This undergoes a Wittig reaction to yield the ester **91**. The ester is reduced to the alcohol **92** with DIBAL-H. The alcohol **92** is oxidized to the aldehyde **93** with DMP (Method a) or TPAP/NMO (Method b). The aldehyde **93** is submitted to a Schlosser's modification[126] of the Wittig reaction to generate the *E*,*E*-diene **94**. The acetal **94** is deprotected to the aldehyde **95**, which undergoes a Horner-Emmons-Wadsworth (HEW) reaction with **97** to generate the triene hexaketide-NAC ester **78**. Compound **97** is generated from bromoacetyl bromide and N-acetylcysteamine to make the thioester **96** which is treated with triethyl phosphite to yield the desired phosphonate **97** (Scheme 24).

Scheme 24: Synthesis of **78**

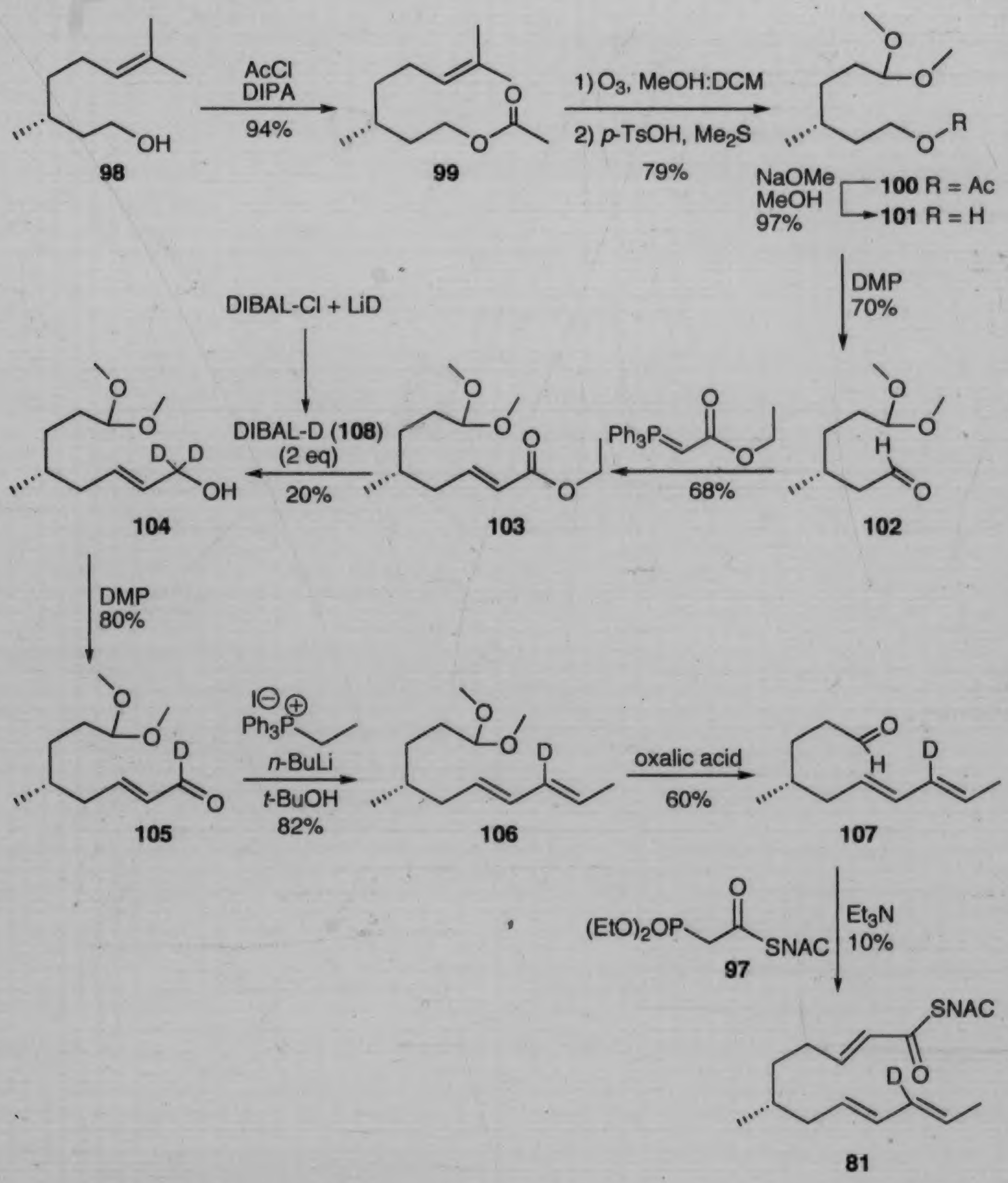

85
LiAlD4
(quant.)
AcCl
DIPA
98%
86 R = H
87 R = Ac
1) O3, MeOH:DCM
2) p-TsOH, Me2S
75%
NaOMe
MeOH
92%
88 R = Ac
89 R = H
DMP
57%
90
Ph3P
93%
91
DIBAL-H
(2 eq)
92%
92
DMP 96%
or
TPAP/NMO 50%
93
Ph3P
n-BuLi
t-BuOH
16%
94
oxalic acid
95%
95
Br
NACSH
16%
96
SNAC
P(OEt)3
49%
(EtO)2OP
97
Et3N
42%
78

The unlabeled **74** and deuterated analogs (**80**-**84**) (Figure 20) can by synthesized in a similar manner starting with *R*-(+)-citronellol (**98**). The deuterium can be incorporated using the appropriate deuterated reductant or Wittig reagent depending on the location of the label.

2.5.3 Synthesis of deuterated analog 81

Incorporation of deuterium into **81** was done by reduction of the dimethoxy alkene ester **103** with DIBAL-D (**108**)[127] to generate the deuterated allylic alcohol **104**. This alcohol was carried through the necessary steps by Dr. Belén Mayo-Martín[128] to yield **81** (Scheme 25).

Scheme 25: Synthesis of **81**

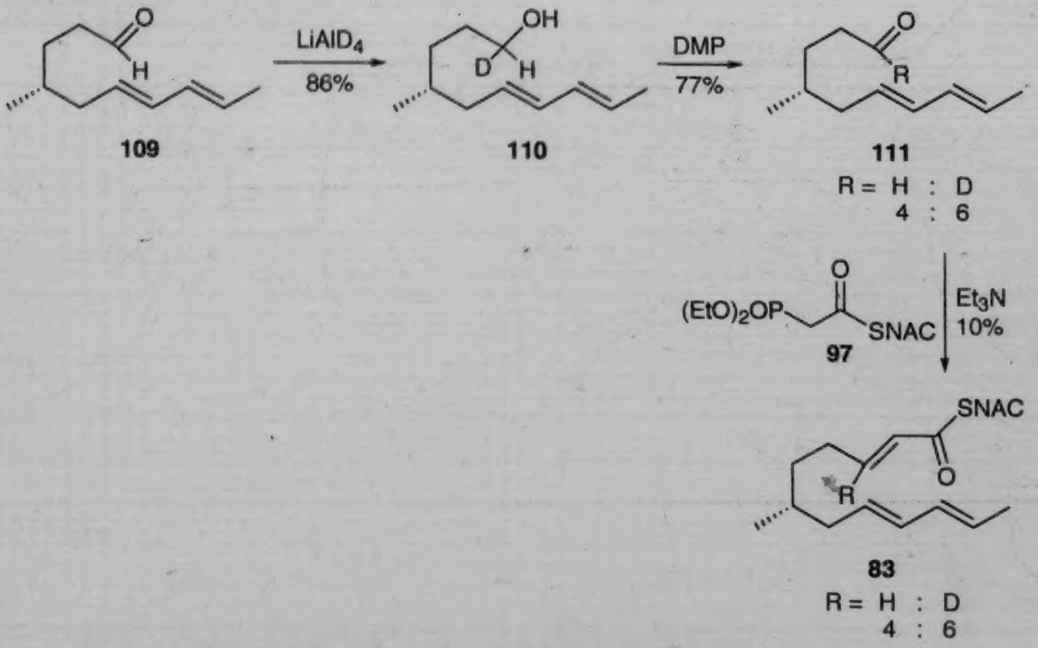

AcCl
DIPA
94%
1) O_3, MeOH:DCM
2) p-TsOH, Me_2S
79%
NaOMe
MeOH
97%
100 R = Ac
101 R = H
DMP
70%
Ph_3P
68%
DIBAL-Cl + LiD
DIBAL-D (108)
(2 eq)
20%
DMP
80%
Ph_3P
n-BuLi
t-BuOH
82%
oxalic acid
60%
$(EtO)_2OP$
SNAC
Et$_3$N
10%
98
99
102
103
104
105
106
107
97
81

2.5.4 Synthesis of deuterated analog 83

The aldehyde **109** and triene **83** were prepared by Dr. Belén Mayo-Martín.[128] The aldehyde **109** was reduced to the alcohol **110** with $LiAlD_4$ followed by oxidation to the aldehyde **111** to incorporate 60% deuterium into the molecule. The mixture underwent a

HEW reaction to yield **83** as a (4:6) mixture of unlabeled and labeled (Scheme 26). This mixture can be used directly in the competition assay to determine the secondary deuterium KIE.

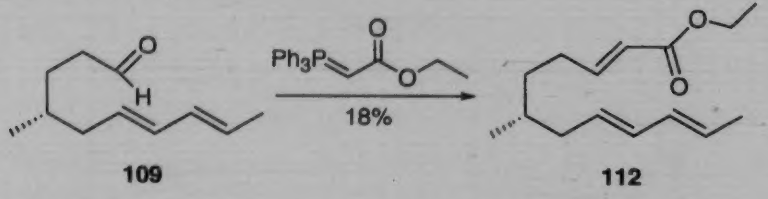
Scheme 26: Synthesis of **83**


LiAlD4
86%
DMP
77%
109
110
111
R = H : D
4 : 6
(EtO)2OP
SNAC
97
Et3N
10%
83
R = H : D
4 : 6

2.5.5 Synthesis of the standard triene hexaketide ethyl ester 112

Compound **112** was synthesized as a standard for ^{1}H-NMR and GC-MS by reaction of **109** with (carbethoxymethylene)triphenylphosphorane (Scheme 27). The low yield obtained may be due to spontaneous cyclization of the product.

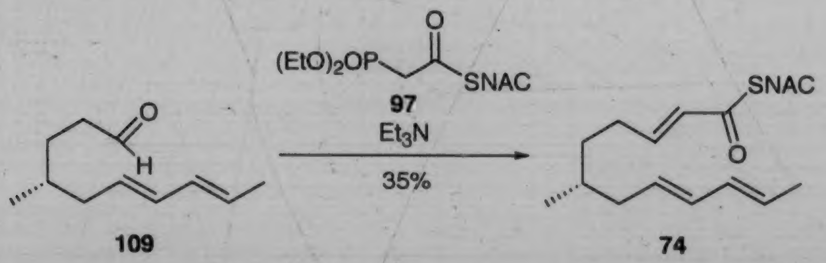
Scheme 27: Synthesis of the triketide ethyl ester **112**


Ph3P
18%
109
112

2.5.6 Synthesis of the triene hexaketide-NAC thioester 74

The non-deuterated triene hexaketide-NAC ester **74** was synthesized in a similar manner to the deuterated analogs above in order to compete with the deuterated version in determining the secondary deuterium KIE (Scheme 28).

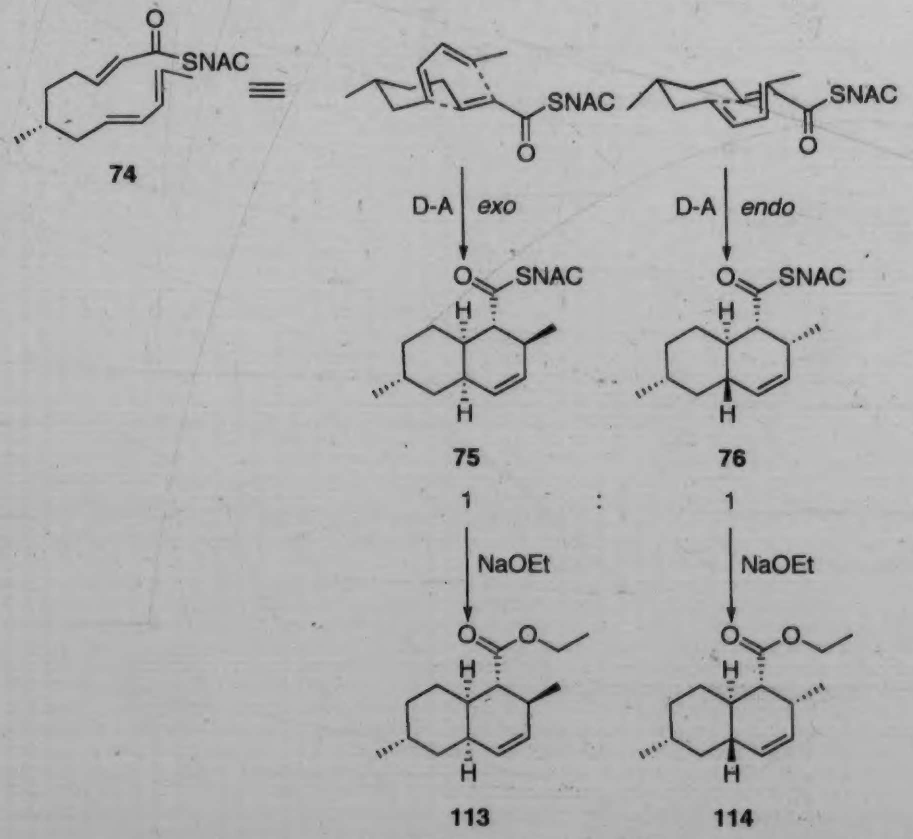
Scheme 28: Synthesis of **74**



2.5.7 Thermal Diels-Alder cyclization of the triene hexaketide-NAC thioester 74

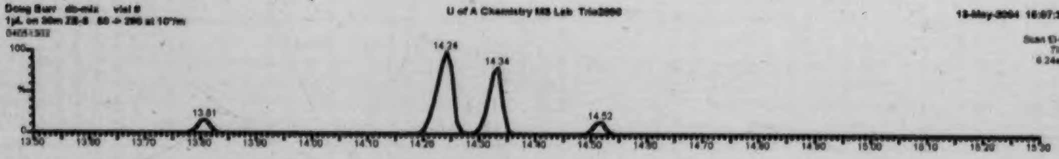
The triene hexaketide-NAC ester **74** was cyclized in pellet buffer (Tris-HCl, sodium ascorbate and DTT, pH 7.8) for 2.5 days at room temperature. As the reaction seemed incomplete by TLC the mixture was heated to 50 °C for a further 2 days. The reaction mixture was extracted and the mixture of diastereomers subjected to ethanolysis to yield the ethyl esters. These were purified as a mixture and subjected to GC-MS. Based on previous work this should yield only the two diastereomers (**113** and **114**) that would form more readily due to their sterically favoured pseudo-equatorial methyl group (Scheme 29 and 21).[73,77] The other diastereomers are not favoured due to the pseudo-axial placement of the methyl group.

Scheme 29: Spontaneous cyclization of the triene hexaketide-NAC ester **74**[52,77]



^{1}H-NMR indicated the presence of the target compounds (**113** and **114**). GC-MS showed two major peaks at 14.25 min and 14.34 min (44% and 53% respectively) with the same correct MW and fragmentation pattern. There were two other small peaks present in the GC-MS at 13.82 min and 14.52 min (1% and 2% respectively) that also showed the same MW and fragmentation pattern as the large peaks indicating the other two diastereomers may be present (Figure 22). GC-MS is a technique that has not been used previously to characterize this reaction and its products. This reaction indicates that LNKS may not be necessary to cyclize **74** *in vitro* to the correct stereochemistry of ring closure present in (**77** and thus **79**) as previously thought. However, it may enhance the yield of **77**. As (**113**, **114** and **79**) had not been characterized by GC-MS, it was necessary to identify each peak in order to make conclusions about this result.

Figure 22: GC trace of the semi-purified ethyl esters from the thermal Diels-Alder cyclization of **74**

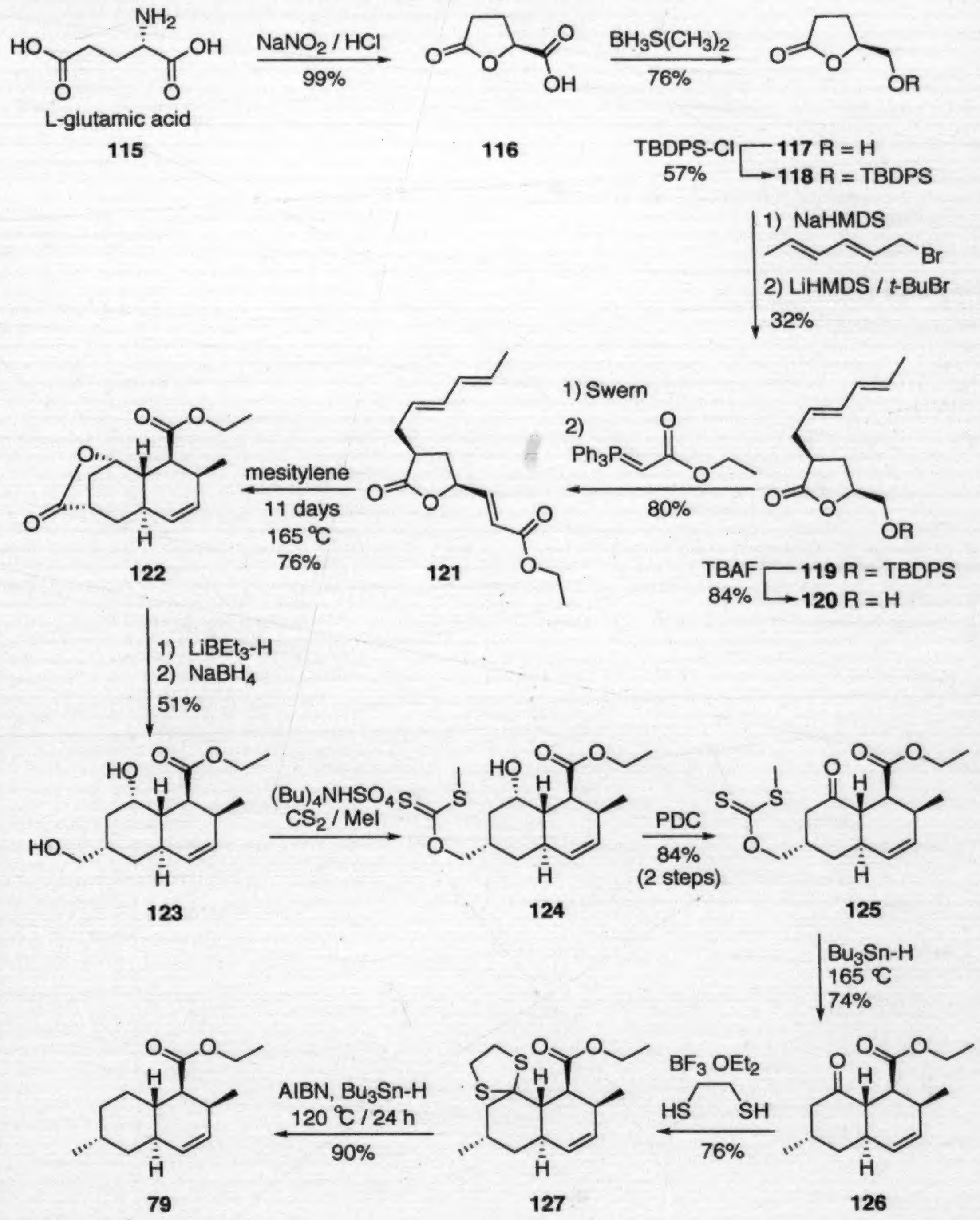


2.5.8 Characterization of GC-MS peaks of the thermal Diels-Alder reaction of 74

The Diels-Alder reaction described above was repeated. The NAC esters **75** and **76** were separated by HPLC and each was subjected separately to ethanolysis and the ethyl esters (**113** and **114**) were isolated. The ^{1}H-NMR spectrum of **114** matched with the literature of the *endo* product,[73] and corresponded to the first large peak on the GC-MS at 14.25 min (Figures 23 and 24). A clean ^{1}H-NMR of the *exo* compound **113** could not be obtained from this reaction, however a standard sample prepared by Dr. Dave Witter[73] was available. GC-MS of this compound identified the second large peak at 14.34 min as **113** as expected (Figures 23 and 24). To determine if **79** is one of the other small peaks present in the GC-MS, a standard was synthesized (Scheme 30).

2.5.9 Synthesis of 79 as a standard for GC-MS

Scheme 30: Synthesis of hexaketide ethyl ester, standard **79**


NH$_2$
HO
OH
L-glutamic acid
115
NaNO$_2$ / HCl
99%
116
BH$_3$S(CH$_3$)$_2$
76%
OR
TBDPS-Cl
57%
117 R = H
118 R = TBDPS
1) NaHMDS
Br
2) LiHMDS / t-BuBr
32%
1) Swern
2)
Ph$_3$P
80%
TBAF
84%
119 R = TBDPS
120 R = H
mesitylene
11 days
165 °C
76%
121
122
1) LiBEt$_3$-H
2) NaBH$_4$
51%
123
(Bu)$_4$NHSO$_4$
CS$_2$ / MeI
124
PDC
84%
(2 steps)
125
Bu$_3$Sn-H
165 °C
74%
126
BF$_3$ OEt$_2$
HS
SH
76%
127
AIBN, Bu$_3$Sn-H
120 °C / 24 h
90%
79

Compound **79** has been previously synthesized in our group and this reaction path was followed (Scheme 30).[73] Successful synthesis of **79** gave the same spectroscopic properties as previously reported.[73] GC-MS of **79** identified the last peak with the same fragmentation pattern as **79**, this had a retention time of 14.52 min (Figures 23 and 24). To verify the identity of each of the three peaks, the crude Diels-Alder mixture that was transesterified and purified together was separated into three aliquots. Each of these aliquots was doped with pure (**113**, **114** or **79**) and within these mixtures the respective peak was enhanced, thereby identifying them unequivocally. The fourth peak with the same fragmentation pattern as (**113**, **114** and **79**) was not identified, but is presumed to be the *exo* ring-closed product with the methyl in the unfavoured pseudo-axial position during cyclization (Scheme 21). This fourth peak had the earliest retention time of 13.82 min.

Figure 23: GC trace of **114** top, **113** middle and **79** bottom.

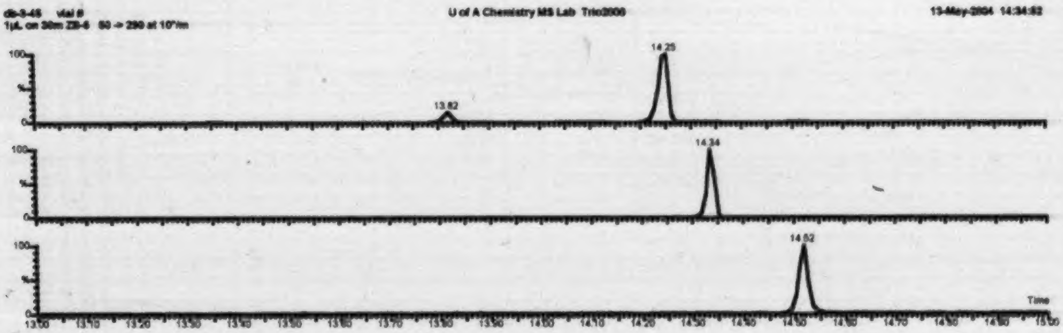
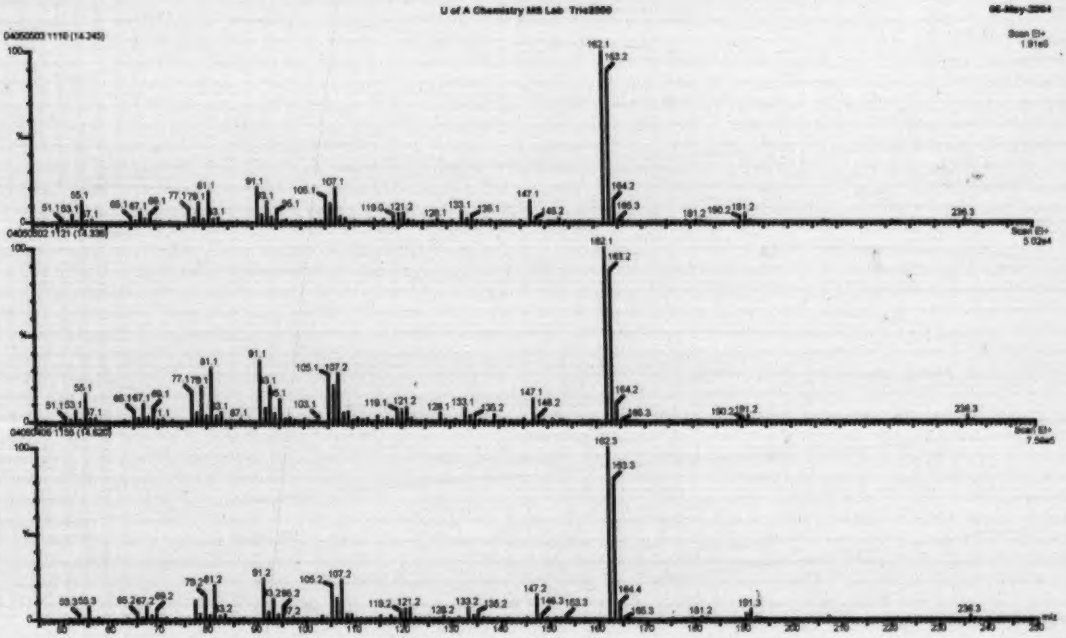


Figure 24: Mass spectrum of **114** top, **113** middle and **79** bottom.



2.6 Secondary deuterium kinetic isotope effects during the Diels-Alder reaction of (74 and 78)

Having identified the expected products by GC-MS, a thermal Diels-Alder reaction was performed using equal amounts of **74** and **78**. The reaction was not allowed to go to completion as this would eliminate the isotope effect. The reactions were quenched, submitted to ethanolysis and purified as a mixture of diastereomers. ^{1}H-NMR identified the presence of the open triene ethyl ester **112** indicating the reaction did not go to completion. The mixture of diastereomers was subjected to GC-MS to identify the correct peaks and to verify that they have the same mass and fragmentation pattern. The three peaks corresponding to **79**, **113** and **114** were identified. The low molecular ion peak using the scanning method prompted use of another GC-MS capable of monitoring selectively at the molecular ions. The EI mass spectrometer was set to scan only 236 and 237 m/z, the molecular weights of the unlabeled and deuterated molecular ions. The GC-MS was run in triplicate to give an average secondary deuterium KIE. In order to remove the contribution from the ^{13}C of the unlabeled compounds 16.7% of the area of the 237 m/z peak was subtracted. The area of the 236 m/z peak was divided by the remaining area of the 237 m/z peak and this number was divided by the ratio of protonated/deuterated starting compounds for each of the three sets of compounds giving an inverse secondary deuterium KIE of 0.933 ± 0.023, 0.934 ± 0.005 and 0.906 ± 0.009 for **79**, **113** and **114** respectively. The inverse KIE is indicative of a concerted rather than stepwise mechanism.[85]

2.7 Significance

Due to previous results, which showed that boiled LovB was unable to catalyze the Diels-Alder reaction of triene NAC thioester **74** to the cyclic NAC thioester **77**[77] and that *in vitro* LovB activity cannot be proven by incorporation studies nor by isolation of radioactive analogs of the pyrones **3** and **4**, further studies involving the mechanism of the LNKS catalyzed Diels-Alder must be deferred. It is known that during previous reactions with **74** to produce **77**, the substrate does not covalently bind to the active site as the product cyclic **77** is isolated as the NAC ester and not an acid. It would therefore be of interest to determine if there is an enhancement in cyclic ethyl ester **79** with LNKS present as compared to an enzyme free system. If there is an enhancement in the amount of **79** produced in the presence of LNKS then it may be possible that the active site can still hold the NAC ester in the correct conformation to enhance the Diels-Alder reaction and mechanistic studies can be undertaken. For a complete mechanistic study the remaining three deuterated analogs would need to be synthesized.

In summary, experiments designed to test the *in vitro* activity of LovB have been performed, and have not revealed information regarding the precursors of the early stages of lovastatin (**2**) biosynthesis. The first synthesis of the pyrones has been demonstrated both in the case of the natural unsaturated (**3** and **4**) and saturated (**18** and **19**) analogs. The more stable methylated versions of each of these (**67**, **37**, **18b** and **32**) have also been prepared. The synthesis of unlabeled **74** and deuterated analogs (**78**, **81** and **83**) of the open hexaketide-NAC esters have been achieved. Using secondary deuterium KIE studies, it appears that the thermal closure of **74** does proceed *via* a concerted mechanism. The study of the mechanism of the ring closure of **74** has led to the use of a more sensitive GC-MS method for analysis and the discovery that **79** is produced by a thermal Diels-Alder reaction of **74**, *albeit* in small quantities (2%). The synthesis of **79** in 13 steps confirmed the identity in the GC trace.

CHAPTER 3. Experimental Procedures

3.1 GENERAL METHODS FOR CHEMICAL SYNTHESES

3.1.1 Reagents, Solvents and Solutions

All reactions involving air or moisture sensitive reactants were done under a positive pressure of dry argon. Solvents were reagent grade and used as supplied unless otherwise stated. For anhydrous reactions, solvents were dried according to Perrin *et al* and Vogel.[129,130] Tetrahydrofuran and diethyl ether were distilled over sodium and benzophenone under an atmosphere of dry argon. Acetonitrile, dichloromethane, methanol, pyridine and triethylamine were distilled over calcium hydride. Removal of solvent was performed under reduced pressure below 40 °C using a Büchi rotary evaporator, followed by evacuation (< 0.1 mm Hg) to constant sample weight. Deionized water was obtained from a Milli-Q reagent water system (Millipore Co., Milford, MA). Unless otherwise specified, solutions of NH_4Cl, $NaHCO_3$, HCl, NaOH, and LiOH refer to aqueous solutions. Brine refers to a saturated aqueous solution of NaCl. All reagents employed were of American Chemical Society (ACS) grade or finer and were used without further purification unless otherwise stated.

3.1.2 Purification Techniques

All reactions and fractions from column chromatography were monitored by thin layer chromatography (TLC) using glass plates with a UV fluorescent indicator (normal SiO_2, Merck 60 F_{254}). One or more of the following methods were used for visualization: UV absorption by fluorescence quenching; iodine staining; (phosphomolybdic acid:ceric sulfate:sulfuric acid:H_2O/10 g:1.25 g:12 mL:238 mL) spray. Flash chromatography was performed according to the method of Still *et al.*[131] using Merck type 60, 230-400 mesh silica gel.

High performance liquid chromatography (HPLC) was performed on either a Beckman System Gold instrument equipped with a model 166 variable wavelength UV detector and a Rheodyne 7725i injector with a 20 to 2000 μL sample loop, on a Rainin instrument equipped with a Rainin model UV-1 variable wavelength UV detector and a Rheodyne 7725i injector fitted with a 1 mL sample loop, on a Gilson instrument equipped with a model 152 variable wavelength UV detector and a Rheodyne 7010 injector fitted with a 1 mL sample loop, or on a Varian prostar equipped with a model 325 variable wavelength UV detector and a Rheodyne 7725i injector fitted with a 20 to 2000 μL sample loop. The columns used were Waters Nova-Pak cartridges (reversed-phase, 8NVC18, 4 μm C_{18} column, 60 Å, 4 μm, 8 x 100 mm), Waters μBondapak cartridges (reversed-phase μBondapak, WAT037684, C_{18} column, 125 Å, 10 μm, 25 x 100 mm), Waters Nova-Pak cartridges (reversed-phase, 8NVPH, 4 μm phenyl column, 60 Å, 4 μm, 8 x 100 mm) or Varian C_{18} steel walled column (reversed-phase, R0086200C5, microsorb-MV100, 5μm C_{18} column, 5 μm, 4.6 x 250 mm). All HPLC solvents were filtered with a Millipore filtration system under vacuum before use. All GC-MS were performed using a Zebron DB5 column of length (30 m) with a stationary phase thickness of (0.24 μm) using the method (50 °C → 250-290 °C @ 10 °C/min).

3.1.3 Instrumentation for compound characterization

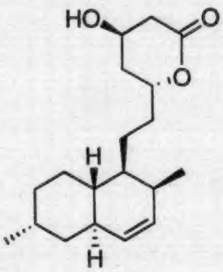
Melting points are uncorrected and were determined on a Thomas-Hoover oil immersion apparatus using open capillary tubes. Optical rotations were measured on a Perkin Elmer 241 polarimeter with a microcell (10 cm, 1 mL) at ambient temperature and are reported in units of 10^{-1} deg cm^2 g^{-1}. All specific rotations reported were referenced against air and were measured at the sodium D line and values quoted are valid within ±1°. Infrared spectra (IR) were recorded on a Nicolet Magna 750 or a 20SX FT-IR spectrometers. Cast refers to the evaporation of a solution on a NaCl plate. High-resolution mass spectra (HRMS) were recorded on a Kratos IMS-50 (high resolution, electron impact ionization (EI), and Micromass ZabSpec Hybrid Sector-TOF positive mode electrospray ionization (ES) instruments. Low-resolution mass spectra (LRMS) were obtained on an HP 1100 (low-resolution electrospray ionization (ES)). Microanalyses were obtained using a Perkin Elmer 240 or Carlo Erba 1108 elemental analyzers.

Nuclear magnetic resonance (NMR) spectra were obtained on Inova Varian 300, 400, 500 and 600 MHz spectrometers. ^1H NMR chemical shifts are reported in parts per million (ppm) downfield relative to tetramethylsilane (TMS) using the residual proton resonance of solvents as reference: $CDCl_3$ δ 7.24, CD_2Cl_2 δ 5.32, D_2O δ 4.72, and CD_3OD δ 3.30. ^{13}C NMR chemical shifts are reported relative to $CDCl_3$ δ 77.0, CD_2Cl_2 δ 53.8, and CD_3OD δ 49.0. Selective homonuclear decoupling, shift correlation spectroscopy (COSY), heteronuclear multiple quantum coherence (HMQC), heteronuclear multiple bond correlation (HMBC) and attached proton test (APT) were used for signal assignments. ^1H NMR data are reported in the following order: multiplicity (s, singlet; d, doublet; t, triplet; q, quartet and m, multiplet), number of protons, coupling constant (*J*) in Hertz (Hz) and assignment. When appropriate, the multiplicity is preceded by br, indicating that the signal was broad. All literature compounds had IR, ^{1}H NMR, and mass spectra consistent with the assigned structures as referenced when applicable.

Radioactivity was determined using standard liquid scintillation procedures in plastic 20 mL scintillation vials (company), with Beckman Ready gel scintillation cocktail (Fullerton, CA). The instrument used was a Beckman LS 5000TD with automatic quench control to determine the decomposition per minute (DPM) in the labeled samples by comparison against a quench curve prepared from Beckman ^{14}C quenched standards.

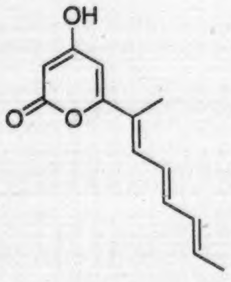
3.2 EXPERIMENTAL DATA FOR COMPOUNDS

Dihydromonacolin L (1)



Dihydromonacolin L (**1**) was isolated from *A. nidulans lovB+C* strain fermented as described in the fermentation section. The culture was vacuum filtered, and the filtrate was acidified (4N HCl, pH 2). The filtrate was then extracted with CH_2Cl_2 (3 X 250 mL). The combined organic layers were washed with brine, dried (Na_2SO_4), filtered and the solvent removed under reduced pressure. The resulting brown oil was purified by flash chromatography (SiO_2, 1:1/hexanes:EtOAc, R_f 0.34) to give a beige solid, which was either used as is or further purified by HPLC (Waters C_{18} column, MeCN and H_2O (0.075% TFA), 15 mL/min, 210 nm), 20% MeCN increasing to 70% MeCN over 20 min then staying there for 5 min before increasing to 100% over 5 min and staying there for 5 min then decreasing to 20% over 0.1 min and staying there for 5 min (t_r 21.5 min). A white solid was obtained that shared the ^{1}H-NMR and mass spectral data as reported by Endo and Hasumi[132] and Vederas and coworkers.[95]

4-Hydroxy-6-[(1*E*,3*E*,5*E*)-1-methylhepta-1,3,5-trien-1-yl]-2*H*-pyran-2-one (3) (Method a)

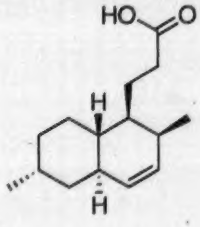


The known compound **3**[52] was prepared using a modified version of the method of Evans and Staunton.[119] TFA (146 µL, 2.03 mmol) and TFAA (146 µL, 1.89 mmol) were added to **47** (55.8 mg, 236 µmol) and the reaction mixture was heated to 70 °C for 45 min. The mixture was cooled and then purified by flash chromatography (SiO_2, 1:1/EtOAc, pentane) to yield pure **3** (8.4 mg, 16%) (SiO_2, 2:1/EtOAc, pentane, R_f 0.13). The product exchanges the proton at C-3 with deuterium of CD_3OD completely after 1 h, but the proton returns if dissolved in CH_3OH. The spectra of the product matched that previously reported by Dr. Karine Auclair.[52] The product is stable if dissolved in MeCN overnight at rt. HPLC (Varian C_{18} column, MeCN and H_2O, 1mL/min, 220 & 360 nm), 30% MeCN to 50% MeCN over 12 min and staying there for 5 min before increasing to 70% over 6 min, then increasing to 100% over 2 min and staying there for 5 min before decreasing to 30% and staying there for 5 min (t_r 17.47 min); IR (CH_2Cl_2 cast) 2927 (br), 1614 (br s), 1542 (s) cm^{-1}; UV (CH_2Cls_2 solution) λ_{max} 365, 274, 206, 202 nm; ^{1}H NMR (CD_3OD, 300 MHz) δ 7.10-7.04 (m, 1H, H-2′), 6.55-6.48 (m, 2H, H-3′ & H-4′), 6.30-6.16 (m, 1H, H-5′), 6.15 (s, 1H, H-5), 5.92 (dq, 1H, J = 13.8, 6.9 Hz, H-6′), 5.37 (d, 1H, J = 2.0 Hz, H-3), 1.97 (s, 3H, 1′-$C\underline{H}_3$), 1.81 (d, 3H, J = 6.9 Hz, H-7′); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 172.8 (C-4), 167.7 (C-2), 163.0 (C-6), 140.5 (C-4′), 134.3 (C-6′), 133.7 (C-2′), 133.3 (C-5′), 126.7 (C-3′), 99.8 (C-5), 90.0 (C-3 & C-1′), 18.6 (C-7′), 12.6 (1′-$\underline{C}H_3$); HRMS (EI) calcd for $C_{13}H_{14}O_3$ 218.0943, found 218.0948 $[M]^+$.

4-Hydroxy-6-[(1*E*,3*E*,5*E*)-1-methylhepta-1,3,5-trien-1-yl]-2*H*-pyran-2-one (3) (Method b)

A modification of the method used by Trauner and coworkers was used.[121] DBU (48 μL, 318 μmol) was added to a stirred solution of **46** (56 mg, 212 μmol) in dry benzene (4 mL) which was then heated to 70 °C for 4 h. The reaction mixture was cooled and the solvent evaporated under reduced pressure. The mixture was purified by flash chromatography, 3 columns (SiO_2, 2:1/EtOAc:pentane) (with CH_2Cl_2 used to help loading), to yield **3** (27 mg, 58%) with the same spectroscopic properties as **3** prepared using Method a above.

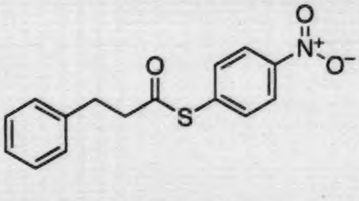
3-[(1*S*,2*S*,4a*R*,6*R*,8a*S*)-2,6-Dimethyl-1,2,4a,5,6,7,8,8a-octahydronaphthalen-1-yl]propanoic acid (6)



The known compound **6**[95] was prepared as follows. 1 M NaOH (100 μL) was added to a stirred solution of the heptaketide-NAC ester **7** (20 mg, 59 μmol) in THF (500 μL) at rt. The mixture was allowed to stir for 4 h before heating to 45 °C for 24 h. There was still starting material present so an additional 50 μL of 1 M NaOH was added and the mixture stirred for 12 h before acidifying (pH 2) and extracting the product with CH_2Cl_2 (3 X 10 mL) to yield the crude acid which was purified by HPLC (Waters C_{18} column, MeCN and H_2O

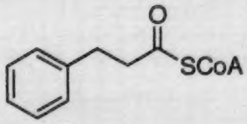
(0.075% TFA), 15 mL/min, 210 nm), 20% MeCN increasing to 70% MeCN over 20 min then staying there for 5 min before increasing to 100% over 5 min and staying there for 5 min then decreasing to 20% over 0.1 min and staying there for 5 min (t_r 22.9 min); $[\alpha]_D^{20}$ +103.21° (c 0.34, $CHCl_3$); IR (microscope) 3200-2500 (br), 3021, 2953, 2915, 2861, 1696, 1463, 1411, 1311 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 5.57 (ddd, 1H, J = 9.9, 4.8, 2.7 Hz, H-3), 5.28 (d, 1H, J = 9.9 Hz, H-4), 2.42 (ddd, 1H, J = 15.6, 10.2, 5.1 Hz, $C\underline{H}_2CO_2H$), 2.30-2.16 (m, 2H), 2.07-1.84 (m, 3H), 1.63-1.32 (m, 6H), 1.27 (ddd, 1H, J = 12.9, 12.9, 4.8 Hz), 1.10 (dddd, 1H, J = 12.0, 12.0, 11.7, 4.5 Hz), 1.04-0.92 (m, 1H), 0.96 (d, 3H, J = 7.2 Hz, 6-$C\underline{H}_3$), 0.83 (d, 3H, J = 7.2 Hz, 2-$C\underline{H}_3$); ^{13}C NMR ($CDCl_3$, 125 MHz) δ; 202.7 ($\underline{C}OOH$), 132.4 ($\underline{C}H$=CH), 131.6 ($\underline{C}H$=CH), 41.0 ($\underline{C}H$), 39.9 ($\underline{C}H$), 38.9 ($\underline{C}H_2$), 37.3 ($\underline{C}H$), 32.3 ($\underline{C}H_2$), 31.9 ($\underline{C}H$), 27.5 ($\underline{C}H$), 23.9 ($\underline{C}H_2$), 23.8 ($\underline{C}H_2$), 23.6 ($\underline{C}H_2$), 18.2 ($\underline{C}H_3$), 14.9 ($\underline{C}H_3$); HRMS (EI) calcd for $C_{15}H_{24}O_2$ 236.1776, found 236.1770 $[M]^+$. The acid **6** was also isolated from a fermentation of LovB+C in the same manner as for the isolation of **1**. From 4 L of culture 1.4 mg of **6** was obtained.

S-(4-Nitrophenyl) 3-phenylpropanethioate (9)



The known compound **9**[133] was prepared using a modification of the method of Padmakumar *et al.*[134] A solution of DCC (82.4 mg, 500 μmol) in dry MeCN (500 μL) was added to a cooled (0 °C) stirred solution of 3-phenyl-propionic acid (50 mg 330 μmol) and *p*-nitrothiophenol (67 mg, 432 μmol) in dry MeCN (400 μL) dropwise. The reaction mixture was allowed to stir and warm to rt overnight before it was filtered and the solvents evaporated under reduced pressure. The mixture was purified by flash chromatography (SiO_2, 4:1/EtOAc:hexanes R_f 0.46) to yield a mixture by ^{1}H-NMR. This mixture was further purified by RP-HPLC (Waters C_{18} column, MeCN and H_2O, 10 mL/min, 254 nm), 60% MeCN to 100% MeCN over 10 min and staying there for 5 min before decreasing to 60% over 1 min and staying there for 4 min (t_r 9.80 min) to yield **9** (43.7 mg, 46%). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.25-8.21 (m, 2H, Ar-H̲), 7.57-7.53 (m, 2H, Ar-H̲), 7.34-7.28 (m, 2H, Ar-H̲), 7.26-7.19 (m, 3H, Ar-H̲), 3.04-3.02 (m, 4H, H-2 & H-3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 194.4 (C-1), 139.4 (Ar-C̲), 136.1 (Ar-C̲), 134.6 (Ar-C̲), 128.6 (Ar-C̲), 128.3 (Ar-C̲), 126.6 (Ar-C̲), 124.4 (Ar-C̲), 123.9 (Ar-C̲), 45.5 (C̲H_2), 31.2 (C̲H_2), 4 carbons not seen due to symmetry; HRMS (EI) calcd for $C_{15}H_{13}NO_3S$ 287.0616, found 287.0621 $[M]^+$.

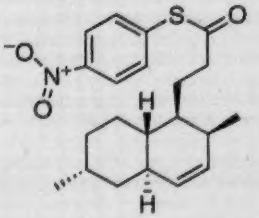
3-Phenyl-thiopropionic acid *S*-coenzymeA ester (10)



The known **10**[135] was prepared using a modification of the method of Chase and Tubbs.[101] $KHCO_3$ (0.1 M, 100 μL) was added to a stirred solution of the thioester **9** (10 mg, 34.8 μmol) and CoASH (26.7 mg, 34.8 μmol) in acetone. The solution turned deep red (as a result of the cleavage of *p*-nitrothioester as the thiolate). The reaction mixture was left to stir for 45 min before acidifying the mixture (pH 2) with conc. HCl (at which point it became colourless). The acetone was then evaporated with a stream of argon. The aqueous layer was washed with Et_2O (10 times) and the residual Et_2O evaporated with a stream of argon. The aqueous layer was then titrated to pH 3.5–4.0 with $KHCO_3$. The mixture was freeze-dried for 5 h to yield **10**. HPLC (Waters C_{18} column, MeOH & H_2O (0.075% TFA), 10 mL/min, 254 nm), 10% MeOH for 15 min then increasing to 70% MeOH over 5 min before increasing to 100%

over 1 min and staying there for 10 min then decreasing to 10% over 1 min and staying there for 8 min (t_r 26.8 min); IR (microscope) 3500-2700 (br), 2965, 1691, 1660, 1539, 1232, 1053, 958 cm^{-1}; LRMS (ES) calcd for $C_{30}H_{44}N_7O_{17}P_3S$ 899.17, found 898.2 [M-H]$^-$, 448.6 [M-2H]$^{-2}$.

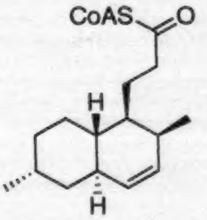
S-(4-Nitrophenyl) 3-[(1*S*,2*S*,4a*R*,6*R*,8a*S*)-2,6-dimethyl-1,2,4a,5,6,7,8,8a-octahydronaphthalen-1-yl]propanethiooate (11)



The same method as for the preparation of **9** was employed. The acid **6** (4.3 mg, 18.2 μmol) was mixed with *p*-nitrothiophenol (4.3 mg, 21.8 μmol) in dry MeCN (300 μL) and DCC (4.5 mg, 21.8 μmol) in dry MeCN (300 μL). After 12 h starting material was still present so additional DCC (4.5 mg) and *p*-nitrothiophenol (4.3 mg) were added and the reaction went to completion. The reaction mixture was purified by RP-HPLC to yield **11** (0.9 mg, 13%) (Waters C_{18} column, Me & H_2O, 15 mL/min, 254 nm), 20% MeCN increasing to 70% MeOH over 20 min then staying there for 5 min before increasing to 100% over 5 min and staying there for 5 min then decreasing to 20% over 0.1 min and staying there for 5 min (t_r 31.7 min). $[\alpha]_D^{20}$ +3.60° (*c* 0.30, $CHCl_3$); IR (microscope) 3325, 3125, 3035, 2928, 2851, 1608, 1539, 1436, 1311, 1242, 1088 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 8.27-8.20 (m, 2H, Ar-CH), 7.62-7.55 (m, 2H Ar-H), 5.58 (ddd, 1H, *J* = 9.6, 4.8, 2.4 Hz, H-3), 5.30 (d, 1H, *J* = 9.9 Hz, H-4), 2.77 (ddd, 1H, *J* = 15.3, 10.2, 5.1 Hz, 1 X C$\underline{H}_2$C(O)S), 2.68-2.53 (m, 1H, 1 X C$\underline{H}_2$C(O)S), 2.30-2.18 (m, 1H), 2.10-1.96 (m, 3H), 1.94-1.85 (m, 1H), 1.32-1.18 (m, 6H), 1.18-1.00 (m, 2H), 0.97 (d, 3H, *J* = 7.2 Hz, 6-

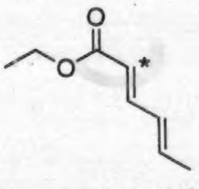
C$\underline{H}_3$), 0.86 (d, 3H, *J* = 6.9 Hz, 2-C$\underline{H}_3$); HRMS (EI) calcd for $C_{21}H_{27}NO_3S$ 373.1712, found 373.1718 $[M]^+$.

3-[(1*S*,2*S*,4a*R*,6*R*,8a*S*)-2,6-Dimethyl-1,2,4a,5,6,7,8,8a-octahydronaphthalen-1-yl]thiopropanoic acid *S*-coenzymeA ester (12)



The same method as for the preparation of **10** was employed. Thus reaction of **11** (7.8 mg, 21.4 μmol) with CoASH (20 mg) and $KHCO_3$ (0.1 M, 400 μL) in acetone (1 mL) for 13 h to yield **12** (5.2 mg, 34%) after purification by HPLC. During the workup, the aqueous solvent was removed under reduced pressure instead of by freeze drying. HPLC (Waters C_{18} column, MeOH & H_2O (0.075% TFA), 10 mL/min, 254 nm), 10% MeOH for 15 min then increasing to 70% MeOH over 5 min before increasing to 100% over 1 min and staying there for 10 min then decreasing to 10% over 1 min and staying there for 8 min (t_r 29.00 min); LRMS (ES) calcd for $C_{36}H_{58}N_7O_{17}P_3S$ 985.28, found 984.3 $[M-H]^-$ & 984.3 $[M-H]^-$, 491.6 $[M-2H]^{-2}$.

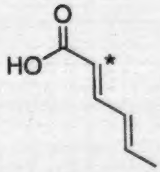
^{13}C labeled ethyl sorbate (15)



The labeled ethyl sorbate (**15**) was prepared by Mr. Kris Rathwell.[102] The labeled ylid was generously donated by Mr. Jamie Côté. A solution of crotonaldehyde (0.85 mL, 10.3 mmol) in dry THF (5 mL) was added to a cooled (0 °C) stirred solution of the ylid (4.91 g, 11.4 mmol) in dry THF (45 mL) and stirred overnight. The solvent was removed under reduced pressure and the mixture purified by flash chromatography, 2 columns (SiO_2, 5% Et_2O in pentane) and (SiO_2, 2% Et_2O in pentane). Due to the volatility of the product the solvent was not completely

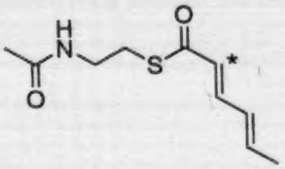
evaporated. IR (CH_2Cl_2 cast) 2981, 2937, 1712, 1643, 1242 cm^{-1}; ^{1}H NMR ($CDCl_3$, 600 MHz) δ 7.22 (ddd, 1H, *J* = 15.3, 10.2, 2.2 Hz, H-3), 6.20-6.07 (m, 2H, H-4 & H-5), 5.75 (dd, 1H, *J* = 162.2, 15.3 Hz, H-2), 4.17 (q, 2H, *J* = 7.1 Hz, H-2′), 1.83 (d, 3H, *J* = 6.1 Hz, H-6), 1.27 (t, 3H, *J* = 7.1 Hz, H-3′); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 145 (d, *J* = 70.2 Hz, C-3), 139 (d, *J* = 9.6 Hz, C-5), 130 (d, *J* = 1.2 Hz, C-4), 119 (m, C-2), 60 (C-2′), 19 (C-6), 14 (C-3′); HRMS (EI) calcd for $^{13}C_1C_7H_{12}O_2$ 141.0870, found 141.0874 $[M]^+$.

^{13}C labeled sorbic acid (16)



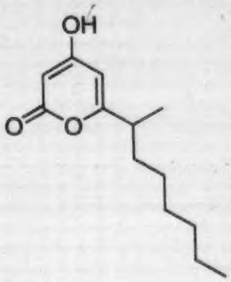
The labeled sorbic acid (**16**) was prepared by Mr. Kris Rathwell.[102] A solution of **15** was added to a cooled (0 °C) stirred solution of KOH (3.06 g, 54.5 mmol) in EtOH (25 mL) and H_2O (7 mL). The reaction mixture was warmed to reflux for 2 h. After cooling the reaction mixture was diluted with H_2O (20 mL), acidified to < pH 4 with 6 N HCl and extracted with Et_2O. The organic layers were combined and neutralized with H_2O, dried (Na_2SO_4) and the solvent evaporated under reduced pressure. The solid was recyrstallized to purity. IR (CH_2Cl_2 cast) 3300-2800 (br), 3050, 2951, 1673, 1636 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.39-7.30 (m, 1H, H-3), 6.28-6.14 (m, 2H, H-4 & H-5), 5.75 (dd, 1H, *J* = 163.2, 15.3 Hz, H-2), 1.83 (d, 3H, *J* = 4.9 Hz, H-6); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 172 (d, *J* = 74.0 Hz, C-1), 147 (d, 69.5 Hz, C-3), 141 (d, *J* = 9.6 Hz, C-5), 130 (d, *J* = 0.9 Hz, C-4), 119 (m, C-2), 19 (C-6); HRMS (EI) calcd for $^{13}C_1C_5H_8O_2$ 113.0557, found 113.0558 $[M]^+$.

^{13}C labeled sorbic acid, *S*-(N-acetylcysteamine) thioester (17)



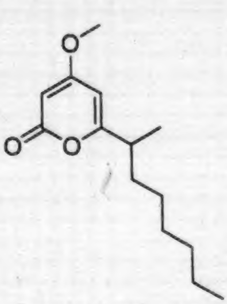
A modification of the method of Vederas and coworkers was used.[63] The labeled triketide NAC thioester **17** was prepared by Mr. Kris Rathwell.[102] A solution of DCC (829 mg, 4.0 mmol) and DMAP (24.6 mg) in dry CH_2Cl_2 (10 mL) along with N-acetylcysteamine (0.42 mL, 3.9 mmol) in dry CH_2Cl_2 (10 mL) were added at the same time to a cooled (0 °C) stirred solution of **16** (388 mg, 3.4 mmol) in dry CH_2Cl_2 (40 mL). After 2 days the reaction mixture was filtered and the solvent removed under reduced pressure. The solid was suspended in EtOAc and the undissolved dicyclohexyl urea was removed by filtration. The solvent was removed under reduced pressure and the crude mixture purified by flash chromatography (SiO_2, 3% MeOH in EtOAc) to yield **17** as a white solid (308 mg, 42%). IR (CH_2Cl_2 cast) 3256, 3071, 2944, 1666, 1635, 1561 cm^{-1}; ^{1}H NMR ($CDCl_3$, 600 MHz) δ 7.17 (dd, 1H, *J* = 15.0, 11.3 Hz, H-3), 6.28-6.12 (m, 2H, H-4 & H-5), 6.07 (dd, 1H, *J* = 161.8, 15.0 Hz, H-2), 6.01 (br s, 1H, N$\underline{H}$), 3.46 (apparent q, 2H, *J* = 6.3 Hz, C$\underline{H}_2$NH), 3.10 (t, 2H, *J* = 6.3 Hz, C$\underline{H}_2$S), 1.96 (s, 3H, $\underline{C}H_3C(O)$), 1.87 (d, 3H, *J* = 6.7 Hz, H-6); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 190 (d, *J* = 63.8 Hz, C-1), 170 ($CH_3\underline{C}(O)$), 142 (d, 69.5 Hz, $\underline{C}$H), 142 (d, *J* = 9.1 Hz, $\underline{C}$H), 130 ($\underline{C}$H), 126 (m, C-2), 40 ($\underline{C}H_2NH$), 28 ($\underline{C}H_2S$), 23 ($\underline{C}H_3C(O)$), 19 (C-6); HRMS (EI) calcd for $^{13}C_1C_9H_{15}NO_2S$ 214.0857, found 214.0855 $[M]^+$.

4-Hydroxy-6-(1-methylheptyl)-2*H*-pyran-2-one (18)



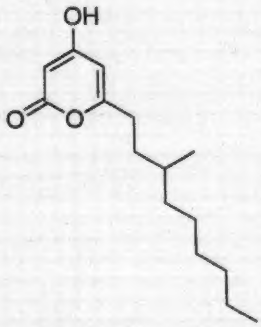
The known compound **18**[52] was prepared using a modification of the method of Poulton and Cyr.[103] *n*-BuLi (1.6 M in hexanes, 21.8 mL, 35 mmol) was added dropwise to a cooled (-78 °C) solution of 4-hydroxy-6-methyl-2-pyrone (2 g, 15.9 mmol) dissolved in dry THF (33 mL) and the mixture turned deep red. The mixture was then warmed to 0 °C and HMPT (7 mL) added. The reaction mixture was stirred for 2 h before 1-bromohexane (2.34 mL, 16.6 mmol) was added and the solution warmed to rt for 18 h. The colour changed from red to orange. The reaction mixture was cooled to -78 °C and *n*-BuLi (1.6 M in hexanes, 10.9 mL, 17.5 mmol) added dropwise, returning the red colour. The reaction mixture was warmed to 0 °C and stirred for 2 h followed by the addition of MeI (1.00 mL, 16.1 mmol). The solution was left to warm to rt over 22 h at which time the colour changed from red to orange. Ice-water (100 mL) was added and the layers separated. The aqueous layer was washed with pentane (3 X 33 mL). The aqueous layer was then acidified (pH 6) with 1 N HCl and extracted with Et_2O (3 X 100 mL). The ethereal layers were washed with H_2O and brine, dried ($MgSO_4$), filtered and the solvent evaporated under reduced pressure. This crude mixture was purified by flash chromatography (SiO_2, 1:1/EtOAc:pentane) to yield **18** as a pure solid (0.3141 g, 9%) (SiO_2, 2:1/EtOAc:pentane, R_f 0.38). The spectra of the product matched that previously reported by Dr. Karine Auclair.[52] The product was stable sitting on the bench open to the air for 2 months (no change by ^{1}H-NMR). HPLC (Varian C_{18} column, MeCN and H_2O, 1 mL/min, 220 nm), 30% MeCN to 50% MeCN over 12 min and staying there for 5 min before increasing to 70% over 6 min then increasing to 100% over 2 min and staying there for 5 min before decreasing to 30% and staying there for 5 min (t_r 23.98 min); IR (microscope) 3150-2500 (br), 2956, 2930, 2858, 2750, 2614, 1692, 1658, 1626, 1567, 1441, 1244 cm^{-1}; ^{1}H NMR (CDCl$_3$, 300 MHz) δ 5.95 (d, 1H, *J* = 2.1 Hz, H-5), 5.56 (d, 1H, *J* = 2.1 Hz, H-3), 2.53 (tq, 1H, *J* = 6.9, 6.9 Hz, H-1′), 1.71-1.56 (m, 1H, 1 X H-2′), 1.51-1.35 (m, 1H, 1 X H-2′), 1.33-1.18 (m, 8H, H-3′ & H-4′ & H-5′ & H-6′), 1.18 (d, 3H, *J* = 6.9 Hz, 1′-C$\underline{H}_3$), 0.84 (t, 3H, *J* = 6.9 Hz, H-7′); ^{13}C NMR (CDCl$_3$, 125 MHz) δ 172.4 (C-4), 171.1 (C-6) 168.2 (C-2), 100.1 (C-5), 89.8 (C-3), 38.4 (C-1′), 34.4 (C-2′), 31.6 (C-5′), 29.1 (C-4′), 27.1 (C-3′), 22.6 (C-6′), 18.1 (1′-$\underline{C}$H$_3$), 14.0 (C-7′); HRMS (EI) calcd for $C_{13}H_{20}O_3$ 224.1412, found 224.1414 [M]$^+$.

4-Methoxy-6-(1-methylheptyl)-2*H*-pyran-2-one (18b)



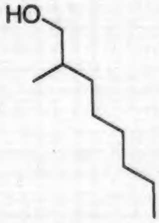
The same method as for the preparation of **27** was employed. Thus, reaction of the **18** (9.8 mg, 43.7 μmol) with K$_2$CO$_3$ (39.3 mg, 284 μmol) and dimethylsulfate (5 μL, 52 μmol) in dry acetone (5 mL) for 1.5 h afforded **18b** after purification by flash chromatography (SiO$_2$, 2:1/EtOAc:pentane, R$_f$ 0.69). HPLC (Varian C$_{18}$ column, MeCN and H$_2$O, 1 mL/min, 220 nm), 30% MeCN to 50% MeCN over 12 min and staying there for 5 min before increasing to 70% over 6 min then increasing to 100% over 2 min and staying there for 5 min before decreasing to 30% and staying there for 5 min (t_r 29.30 min); IR (microscope) 3088, 2929, 2857, 1727, 1649, 1569, 1455, 1412, 1246 cm^{-1}; ^{1}H NMR (CDCl$_3$, 400 MHz) δ 5.72 (d, 1H, *J* = 2.4 Hz, H-5), 5.37 (d, 1H, *J* = 2.4 Hz, H-3), 3.78 (s, 3H, OC$\underline{H}_3$), 2.46 (tq, 1H, *J* = 6.8, 6.8 Hz, H-1′), 1.71-1.60 (m, 1H, 1 X H-2′), 1.48-1.38 (m, 1H, 1 X H-2′), 1.31-1.18 (m, 8H, H-3′ & H-4′ & H-5′ & H-6′), 1.18 (d, 3H, J = 6.8 Hz, 1′-CH$_3$), 0.85 (t, 3H, J = 6.8 Hz, H-7′); ^{13}C NMR (CDCl$_3$, 100 MHz) δ 171.3 (C-4), 169.7 (C-6) 165.1 (C-2), 98.6 (C-5), 87.5 (C-3), 55.7 (OCH$_3$) 38.3 (C-1′), 34.3 (C-2′), 31.7 (C-5′), 29.1 (C-4′), 27.1 (C-3′), 22.6 (C-6′), 18.2 (1′-CH$_3$), 14.0 (C-7′); HRMS (EI) calcd for $C_{14}H_{22}O_3$ 238.1569, found 238.1564 [M]$^+$.

4-Hydroxy-6-(3-methylnonyl)-2*H*-pyran-2-one (19)



The known compound **19**[52] was prepared using a modification of the method of Olah *et al.*[114] Anhydrous AlCl$_3$ (55.5 mg, 420 μmol) was added to a stirred solution of the methylated pyrone **32** (22.2 mg, 83 μmol) in dry benzene (3 mL) and the reaction fitted with a condenser and refluxed for 3 h. The solvent was evaporated under reduced pressure and 2 N HCl (10 mL) was added at 0 °C followed by solid NaHCO$_3$ until the mixture came to pH 3.5-4, it was then diluted with H$_2$O and extracted with EtOAc (3 X 20 mL). The organic fractions were dried (MgSO$_4$), filtered and the solvent evaporated under reduced pressure. The crude mixture was purified by flash chromatography (SiO$_2$, 2:1/EtOAc:pentane, R$_f$ 0.17) to yield **19** (15.5 mg, 74%) as a beige oil which solidified upon cooling. The spectra of the product matched that previously reported by Karine Auclair.[52] The product is stable dissolved in MeCN overnight at rt. HPLC (Varian C$_{18}$ column, MeCN and H$_2$O, 1mL/min, 220 & 280 nm), 30% MeCN to 50% MeCN over 12 min and staying there for 5 min before increasing to 70% over 6 min then increasing to 100% over 2 min and staying there for 5 min before decreasing to 30% and staying there for 5 min (t_r 29.28 min); UV (MeCN solution) λ_{max} 288, 202 nm; IR (microscope) 3200-2500 (br), 3083, 2956, 2926, 2856, 2737, 2623, 1659, 1624, 1567, 1492, 1443, 1378, 1291, 1252 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 5.97 (d, 1H, *J* = 2.0 Hz, H-5), 5.56 (d, 1H, *J* = 2.0 Hz, H-3), 2.53-2.38 (m, 2H, H-1′), 1.68-1.57 (m, 1H, 1 X H-2′), 1.46-1.37 (m, 2H, 1 X H-2′ & H-3′), 1.30-1.16 (m, 9H, 1 X H-4′ & H-5′ & H-6′ & H-7′ & H-8′), 1.14-1.07 (m, 1H, 1 X H-4′), 0.87-0.83 (m, 6H, 3′-$\underline{C}H_3$ & C-9′); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 172.7 (C-4), 168.3 (C-2), 167.8 (C-6), 101.2 (C-5), 89.7 (C-3), 36.6 (C-1′), 33.8 (C-6′), 32.3 (C-3′), 31.9 ($\underline{C}H_2$), 31.4 ($\underline{C}H_2$), 29.6 (C-7′), 26.8 (C-2′), 22.7 (C-8′), 19.3 (3′-$\underline{C}H_3$), 14.1 (C-9′); HRMS (EI) calcd for $C_{15}H_{24}O_3$ 252.1725, found 252.1719 [M]$^+$.

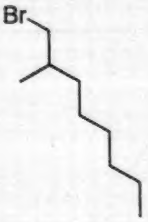
2-Methyloctan-1-ol (23)



The known compound **23**[104] was prepared using a modification of the method of Sabadie and Descotes.[104] Sodium metal (17 g, 740 mmol) was added portion-wise to a cooled (0 °C) mixture of octanol (**22**) (31.5 mL, 200 mmol) and MeOH (101.4 mL, 2.5 mol). To this mixture was added 10% Pd/C (0.7 g) and the mixture placed in an autoclave (200 °C, 1000 psi.) for 6 h, before cooling and depressurizing overnight. The mixture was diluted with H_2O and Et_2O and acidified to pH 3-4 with 4 N HCl. The layers were separated and the aqueous layer extracted with Et_2O (thrice). The ethereal layers were combined, dried (Na_2SO_4), filtered and the solvent evaporated under reduced pressure. The crude mixture was distilled to get rid of some impurities. The fractions which contained the product were purified by flash chromatography (SiO_2, 2:8/Et_2O:pentane, R_f 0.24) to yield **23** (8.6853 g, 30%) as a colourless liquid. IR (Neat) 3331 (br s), 2956, 2924, 2856, 1466, 1378, 1034 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 3.49 (dd, 1H, *J* = 10.5, 6.0 Hz, 1 X H-1), 3.39 (dd, 1H, *J* =

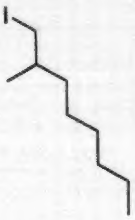
10.5, 6.6 Hz, 1 X H-1), 1.66-1.52 (m, 1H, H-2), 1.44-1.20 (m, 9H, 1 X H-3 & H-4 & H-5 & H-6 & H-7), 1.15-1.02 (m, 1H, 1 X H-3), 0.91-0.83 (m, 6H, 2-CH$_3$ & H-8); ^{13}C NMR (CDCl$_3$, 75 MHz) δ 68.3 (C-1), 35.7 (C-2), 32.5 (CH$_2$), 32.0 (CH$_2$), 29.6 (CH$_2$), 26.8 (CH$_2$), 22.4 (CH$_2$), 16.7 (2-CH$_3$), 14.1 (C-8); HRMS (EI) calcd for $C_9H_{20}O$ 144.1514, found 144.1514 $[M]^+$.

1-Bromo-2-methyloctane (24)



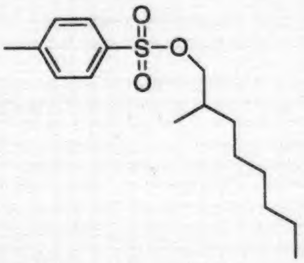
The known compound **24**[136] was prepared using a modification of the method of Wiley *et al.*[137] A solution of PBr_3 (280 μL, 2.97 mmol) in dry Et_2O (6 mL) was added to a cooled (-10 °C) stirred solution of **23** (1.3 g, 9 mmol) in dry Et_2O (6 mL) and the reaction mixture left to stir for 45 min before diluting with Et_2O and H_2O. The layers were separated and the ethereal layer was dried ($MgSO_4$), filtered and the solvent evaporated under reduced pressure. The mixture was purified by flash chromatography (SiO_2, 2:8/Et_2O:pentane, R_f 0.94) to yield the bromide **24** (258 mg, 14%). The major side product (about 40%) was a phosphate. IR (Neat) 2957, 2926, 2871, 2855, 1458, 1378, 1230 cm^{-1}; ^{1}H NMR (CDCl$_3$, 300 MHz) δ 3.38 (dd, 1H, *J* = 9.6, 4.8 Hz, 1 X H-1), 3.30 (dd, 1H, *J* = 9.6, 6.3 Hz, 1 X H-1), 1.84-1.69 (m, 1H, H-2), 1.50-1.36 (m, 1H, 1 X H-3), 1.34-1.16 (m, 9H, 1 X H-3 & H-4 & H-5 & H-6 & H-7), 0.99 (d, 3H, *J* = 6.6 Hz, 2-CH$_3$), 0.87 (t, 3H, *J* = 6.9 Hz, H-8); ^{13}C NMR (CDCl$_3$, 125 MHz) δ 41.5 (C-1), 35.2 (C-2), 34.9 (CH$_2$), 31.8 (CH$_2$), 29.4 (CH$_2$), 26.8 (CH$_2$), 22.6 (CH$_2$), 18.8 (2-CH$_3$), 14.1 (C-8); HRMS (EI) calcd for $C_9H_{19}Br$ 206.0670, found 126.1405 $[M-HBr]^+$.

1-Iodo-2-methyloctane (25)



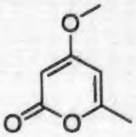
The racemic form of the known compound **25**[138] was prepared using a modification of the method of Pearson and Lee.[107] NaI (175 mg, 1.17 mmol) was added to a stirred solution of **24** (48.3 mg, 233 µmol) in dry acetone (15 mL) and the reaction mixture stirred at rt for 45 min before fitting with a condenser and refluxing overnight. The reaction mixture was cooled, and the solid filtered (washing with acetone) and the solvent evaporated under reduced pressure. The mixture was dissolved in CH_2Cl_2 and washed with brine. The brine layer was back extracted with CH_2Cl_2, the organic fractions were combined, dried ($MgSO_4$), filtered and the solvent removed under reduced pressure. The mixture was purified by flash chromatography (SiO_2, pentane, R_f 0.80) to yield **25** (39.8 mg, 67%). IR (microscope) 2957, 2925, 2854, 1458, 1377, 1193 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 3.24 (dd, 1H, *J* = 9.4, 4.5 Hz, 1 X H-1), 3.15 (dd, 1H, *J* = 9.4, 6.0 Hz, 1 X H-1), 1.50-1.40 (m, 1H, H-2), 1.40-1.16 (m, 10H, H-3 & H-4 & H-5 & H-6 & H-7), 0.98 (d, 3H, *J* = 6.3 Hz, 2-$C\underline{H}_3$), 0.89 (t, 3H, *J* = 6.9 Hz, C-8); ^{13}C NMR ($CDCl_3$, 150 MHz) δ; 36.5 ($\underline{C}H_2$), 34.4 (C-2), 31.8 ($\underline{C}H_2$), 29.3 ($\underline{C}H_2$), 26.4 ($\underline{C}H_2$), 22.1 ($\underline{C}H_2$), 17.9 (C-1), 20.5 (2-$\underline{C}H_3$), 14.2 (C-8); HRMS (EI) calcd for $C_9H_{19}I$ 254.0531, found 254.0518 $[M]^+$.

2-Methyloctyl 4-methylbenzenesulfonate (26)



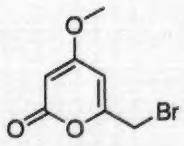
The racemic form of the known compound **26**[139] was prepared using a modification of the method of Fife *et al.*[108] Et_3N (193 µL, 1.39 mmol) was added to a solution of **23** (200 mg, 1.39 mmol) in dry CH_2Cl_2 (1 mL) and the reaction mixture stirred at rt for 20 min. This solution was added dropwise to a cooled (0 °C) stirred solution of Ts-Cl (291 mg, 1.53 mmol) in dry CH_2Cl_2 (1 mL) and the reaction mixture warmed to rt and stirred for 2 days. The mixture was diluted with CH_2Cl_2 and washed with H_2O (twice). The organic fractions were dried ($MgSO_4$), filtered and the solvent evaporated to give a crude mixture of product **26** and starting materials Ts-Cl, and **23**. The mixture was purifed by flash chromatography (SiO_2, 1:9/Et_2O:pentane) to give 393.2 mg of a mixture of product and starting material Ts-Cl which elute together. Using only pentane the Ts-Cl moves by TLC and the product does not, so these two were further purified by flash chromatography (SiO_2, pentane) to elute the Ts-Cl, followed by elution of the product with Et_2O to yield **26** (246 mg, 59%) (SiO_2, 2:8/Et_2O:pentane, R_f 0.51). IR (microscope) 2958, 2928, 2857, 1598, 1495, 1466, 1362, 1189, 1177 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 7.79-7.74 (m, 2H, Ar-H), 7.34-7.29 (m, 2H, Ar-H), 3.86 (dd, 1H, $J = 9.6, 6.0$ Hz, 1 X H-1), 3.78 (dd, 1H, $J = 9.6, 6.3$ Hz, 1 X H-1), 2.45 (s, 3H, Ar-C$\underline{H}_3$), 1.80-1.68 (m, 1H, H-2), 1.34-1.14 (m, 9H, 1 X H-3 & H-4 & H-5 & H-6 & H-7), 1.14-1.02 (m, 1H, 1 X H-3), 0.88-0.82 (m, 6H, 2-C$\underline{H}_3$ & H-8); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 144.6 (*ispo*-$\underline{C}$), 133.2 (*ispo*-$\underline{C}$), 129.8 (Ar-$\underline{C}$H), 127.9 (Ar-$\underline{C}$H), 75.1 (C-1), 32.8 (C-2), 32.7 ($\underline{C}H_2$), 31.7 ($\underline{C}H_2$), 29.3 ($\underline{C}H_2$), 26.5 ($\underline{C}H_2$), 22.6 ($\underline{C}H_2$), 21.6 (Ar-$\underline{C}H_3$), 16.4 (2-$\underline{C}H_3$), 14.0 (C-8), 2 carbons not seen due to symmetry; HRMS (EI) calcd for $C_{16}H_{26}O_3Si$ 298.1603, found 298.1600 $[M]^+$.

4-Methoxy-6-methyl-2*H*-pyran-2-one (27)



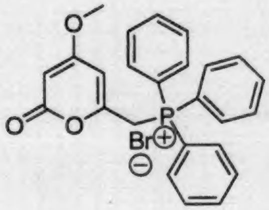
A modification of the method of Poulton and Cyr was used[103] to make the known compound **27.**[140] K_2CO_3 (14.25 g, 51.5 mmol) was added to a vigorously stirred solution of 4-hydroxy-6-methyl-2-pyrone (**20**) (2 g, 15.8 mmol) in dry acetone (50 mL) and left to react for 30 min. Dimethylsulfate (1.52 mL, 19.03 mmol) was added dropwise and the reaction mixture stirred for 2 h. The mixture was then filtered and the solid rinsed with acetone. The solvent was removed under reduced pressure. The crude solid was purified by flash chromatography (SiO_2, 2:1/EtOAc:pentane, R_f 0.47) to yield pure **27** as a white solid (1.9837 g, 89%), mp 82-85 °C (lit.[140] mp 87.5-88.5 °C). IR (microscope) 3085, 3021, 2998, 2957, 1741, 1722, 1653, 1572, 1465, 1407, 1326, 1252 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 5.75-5.72 (m, 1H, H-5), 5.34 (d, 1H, *J* = 2.1 Hz, H-3), 3.77 (s, 3H, OCH_3), 2.14 (s, 3H, C-6); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 171.3 (C-4), 164.9 (C-2), 162.0 (C-6), 100.3 (C-5) 87.3 (C-3) 55.8 ($O\underline{C}H_3$), 19.8 (6-$\underline{C}H_3$); HRMS (EI) calcd for $C_7H_80_3$ 140.0473, found 140.0474 $[M]^+$.

6-(Bromomethyl)-4-methoxy-2*H*-pyran-2-one (28)



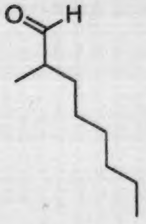
A modification of the method of De March *et al* was used[141] to make the known compound **28.**[109] AIBN (218 mg, 1.33 mmol) and NBS (2.601 g, 14.6 mmol) were added to a stirred solution of **27** (1.8617 g, 13.3 mmol) in dry CCl_4, the reaction mixture was then brought to reflux and stirred in the dark overnight. The solvent was evaporated under reduced pressure to yield 3.6763 g of crude product. A small portion of this was purified by flash chromatography (SiO_2, 1:1/EtOAc:pentane) for characterization. As only the desired brominated product would react with PPh_3, the remainder was carried forward without purification. (SiO_2, 2:1/EtOAc:pentane, R_f 0.44), mp 88-90 °C (lit.[109] mp 93-95 °C). IR (microscope) 3091, 3037, 2979, 2946, 2849, 1722, 1651, 1568, 1455, 1410, 1334, 1257 cm^{-1}; ^1H NMR ($CDCl_3$, 600 MHz) δ 6.07-6.05 (m, 1H, H-5), 5.47-5.44 (m, 1H, H-3), 4.09-4.06 (m, 2H, H-1'), 3.80-3.77 (m, 3H, OCH$_3$); ^{13}C NMR ($CDCl_3$, 150 MHz) δ 170.3 (C-4), 163.1 (C-2), 158.6 (C-6), 102.3 (C-5), 89.3 (C-3), 56.2 (OCH$_3$), 26.5 (C-1'); HRMS (EI) calcd for $C_7H_7BrO_3$ 219.9558, 217.9579, found 219.9558, 217.9575 $[M]^+$.

6-(4-Methoxy-2*H*-pyran-2-one)-methyl-triphenylphosphonium bromide (29)



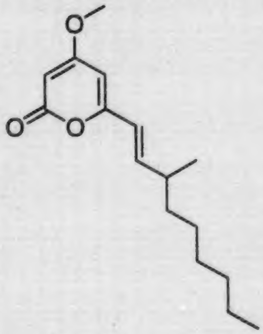
A modification of the method of Bloomer *et al* was used[109] to make the known compound **29**.[109] Triphenylphosphine (3.0 g, 13.3 mmol) was added to a stirred solution of crude **28** in dry toluene (50 mL) and the mixture was brought to reflux and stirred for 20 h. The mixture turned a dark brown colour with sediment present. The reaction mixture was cooled and the solvent evaporated. The solid was recrystallized with EtOH to yield **29** (3.9536g, 62% for 2 steps) as a stable dark brown solid with a (dec) 225-228 °C (lit. 224-226 °C (dec)). IR (CH_2Cl_2 cast) 3040, 2823, 2752, 1723, 1648, 1564, 1436, 1250, 1109 cm^{-1}; UV (CH_2Cl_2 solution) λ_{max} 277, 221 nm;^1H NMR ($CDCl_3$, 300 MHz) δ 7.94-7.83 (m, 6H, Ar-H), 7.83-7.73 (m, 3H, Ar-H), 7.73-7.63 (m, 6H, Ar-H), 7.08 (apparent dd, 1H, *J* = 3.0, 2.3 Hz, H-5), 5.59 (apparent d, 2H, *J* = 14.7 Hz, H-1'), 5.28 (apparent t, 1H, *J* = 2.3 Hz, H-3), 3.69 (s, 3H, OCH$_3$); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 170.8 (C-4), 162.8 (C-2), 152.3, (C-6), 135.4 (d, *J* = 2.6 Hz, Ar-CH), 134.2 (d, *J* = 10.3 Hz, Ar-CH), 130.3 (d, *J* = 12.9 Hz, Ar-CH), 117.4 (d, *J* = 86.6 Hz, *ipso*-C), 107.1 (C-5), 89.1 (C-3), 56.2 (OCH$_3$), 29.5 (d, *J* = 51.0 Hz, C-1'), 14 carbons not seen due to symmetry; HRMS (ES) calcd for $C_{25}H_{22}O_3P$ 401.1301, found 401.1300 $[M]^+$.

2-Methyloctanal (30)



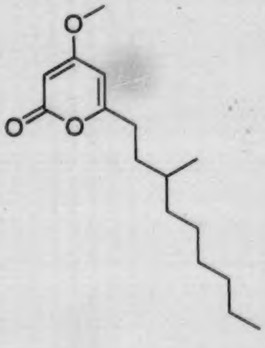
The known compound **30**[142] was prepared as follow. The same method as for the preparation of **102** was employed. Thus, reaction of the **23** (400 mg, 2.77 mmol) in dry CH_2Cl_2 (5 mL) with DMP (1.294 g) in dry CH_2Cl_2 (20 mL) for 22 h afforded **30** (370.7 mg, 94%) after purification by flash chromatography (SiO$_2$, 2:8/Et$_2$O:pentane, R_f 0.65). IR (microscope) 2957, 2929, 2859, 2657, 1708 cm^{-1}; ^{1}H NMR (CDCl$_3$, 300 MHz) δ 9.59 (d, 1H, *J* = 2.1 Hz, H-1), 2.36-2.24 (m, 1H, H-2), 1.76-1.62 (m, 1H, 1 X H-3), 1.36-1.23 (m, 9H, 1 X H-3 & H-4 & H-5 & H-6 & H-7), 1.07 (d, 3H, *J* = 6.9 Hz, 2-CH$_3$), 0.86 (t, 3H, *J* = 6.9 Hz, C-8); ^{13}C NMR (CDCl$_3$, 125 MHz) δ 209.1 (C-1), 37.2 (C-2), 33.6, (CH$_2$), 31.9 (CH$_2$), 31.1 (CH$_2$), 29.6 (CH$_2$), 22.6 (CH$_2$), 16.9 (2-CH$_3$), 14.1 (C-8); HRMS (EI) calcd for $C_9H_{18}O$ 142.1358, found 158.1310 $[M+O]^+$.

4-Methoxy-6-[(1*E*)-3-methylnon-1-en-1-yl]-2*H*-pyran-2-one (31)



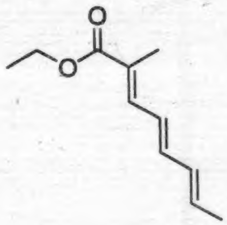
A modification of the method of Bloomer *et al* was used.[109] *n*-BuLi (1.6 M in hexanes, 1.44 mL, 2.3 mmol) was added to a stirred suspension of the Wittig salt **29** (1.007 g, 2.09 mmol) in dry THF (15 mL) at rt and the mixture allowed to stir for 2.5 h. A solution of the aldehyde (270.7 mg, 1.9 mmol) in dry THF (3 mL) was added and the reaction mixture stirred for 17 h. The mixture was diluted with EtOAc and washed with H_2O (2 X 20 mL) and brine (20 mL). The aqueous fractions were combined and back extracted with EtOAc (3 X 20 mL), the organic fractions were combined, dried ($MgSO_4$), filtered and the solvent evaporated under reduced pressure. The mixture was purified by flash chromatography (SiO_2, 2:1/EtOAc, pentane, R_f 0.66) to yield **31** (229.3 mg, 46%). ^{1}H NMR ($CDCl_3$, 600 MHz) δ 6.59, (dd, 1H, *J* = 15.6, 8.4 Hz, H-2′), 5.87 (dd, 1H, *J* = 15.6, 1.2 Hz, H-1′), 5.74 (d, 1H, *J* = 1.8 Hz, H-5), 5.41 (d, 1H, *J* = 1.8 Hz, H-3), 3.78 (s, 3H, $OC\underline{H}_3$), 2.25 (dtq, 1H, *J* = 6.6, 6.6, 6.6 Hz, H-3′), 1.37-1.31 (m, 2H, H-4′), 1.29-1.20 (m, 8H, H-5′ & H-6′ & H-7′ & H-8′), 1.02 (d, 3H, *J* = 6.6 Hz, 3′-$C\underline{H}_3$), 0.85 (t, 3H, *J* = 6.6 Hz, H-9′); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 171.3 (C-4), 164.3 (C-2), 158.9 (C-6), 145.8 (C-2′), 119.6 (C-1′), 99.5 (C-5), 88.2 (C-3), 55.8 ($O\underline{C}H_3$), 37.1 (C-3′), 36.5 (C-4′), 31.8 ($\underline{C}H_2$), 29.3 ($\underline{C}H_2$), 27.3 ($\underline{C}H_2$), 22.6 (C-8′), 19.9 (3′-$\underline{C}H_3$), 14.1 (C-9′); HRMS (EI) calcd for $C_{16}H_{24}O_3$ 264.1725, found 264.1722 $[M]^+$.

4-Methoxy-6-(3-methylnonyl)-2*H*-pyran-2-one (32)



A spatula tip of 10% Pd/C was added to a solution of **31** (170 mg, 643 μmol) in EtOAc (10 mL). The reaction mixture was flushed with H_2 gas, and the mixture put under an atmospheric pressure of H_2 gas overnight. The reaction mixture was then filtered through a bed of celite, washing with EtOAc to yield **31** in quantitative yield as a beige oil that solidified upon freezing to -20 °C. Product is stable dissolved in MeCN overnight at rt. Appeared as one spot by TLC and pure by ^{1}H-NMR. HPLC (Varian C_{18} column, MeCN and H_2O, 1mL/min, 220 & 280 nm), 30% MeCN to 50% MeCN over 12 min and staying there for 5 min before increasing to 70% over 6 min then increasing to 100% over 2 min and staying there for 5 min before decreasing to 30% and staying there for 5 min (t_r 32.00 min); UV (MeCN solution) λ_{max} 282, 208 nm; ^{1}H NMR (CDCl$_3$, 500 MHz) δ 5.73 (d, 1H, J = 2.0 Hz, H-5), 5.36 (d, 1H, J = 2.0 Hz, H-3), 3.75 (s, 3H, OC$\underline{H}_3$), 2.47-2.32 (m, 2H, H-1′), 1.67-1.57 (m, 1H, 1 X H-2′), 1.45-1.36 (m, 2H, 1 X H-2′ & H-3′), 1.30-1.16 (m, 9H, 1 X H-4′ & H-5′ & H-6′ & H-7′ & H-8′), 1.14-1.07 (m, 1H, 1 X H-4′), 0.87-0.82 (m, 6H, 3′-$\underline{C}$H$_3$ & C-9′); ^{13}C NMR (CDCl$_3$, 125 MHz) δ 171.3 (C-4), 166.2 (C-2), 165.0 (C-6), 99.4 (C-5), 87.4 (C-3), 55.7 (O$\underline{C}$H$_3$), 36.6 (C-1′), 33.8 (C-6′), 32.3 (C-3′), 31.8 ($\underline{C}$H$_2$), 31.4 ($\underline{C}$H$_2$), 29.5 (C-7′), 26.8 (C-2′), 22.6 (C-8′), 19.3 (3′-$\underline{C}$H$_3$), 14.1 (C-9′); HRMS (EI) calcd for $C_{16}H_{26}O_3$ 266.1882, found 266.1884 [M]$^+$.

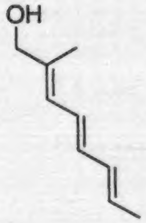
Ethyl (2*E*,4*E*,6*E*)-2-methylocta-2,4,6-trienoate (34)



The known compound **34**[143] was prepared as follows. (2*E*,4*E*)-Hexa-2,4-dienal (795 mg, 8.28 mmol) in dry CH$_2$Cl$_2$ (3 mL) was added to a cooled (0 °C) stirred solution of the ylid (carbethoxyethylidene)triphenylphosphorane (3.00 g, 7.78 mmol, as only 94% pure) in dry CH$_2$Cl$_2$ (30 mL). The reaction mixture was left to stir and warm to rt for 2 days before the solvent was evaporated under reduced pressure. The mixture was then purified by flash chromatography, 2 columns to separate *E*/*Z*, (loading with CH$_2$Cl$_2$ for column #1 only) (SiO$_2$, 5:95/Et$_2$O:pentane) to yield **34** (1.4 g, 99%) with a minor (5%) amount of *Z* compound incorporated from the starting commercial aldehyde. (SiO$_2$, 1:9/Et$_2$O:pentane, R$_f$ 0.60). IR (microscope) 3026, 2981, 2933, 1703, 1641, 1613, 1585

cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 7.17 (dd, 1H, *J* = 11.3, 1.4 Hz, H-3), 6.47 (dd, 1H, *J* = 14.8, 10.5 Hz, H-5), 6.34 (dd, 1H, *J* = 14.8, 11.3 Hz, H-4), 6.17 (ddq, 1H, *J* = 15.0, 10.5, 1.4 Hz, H-6), 5.87 (dq, 1H, *J* = 15.0, 6.9 Hz, H-7), 4.18 (q, 2H, *J* = 7.2 Hz, H-2′), 1.92 (d, 3H, *J* = 1.4 Hz, 2-C$\underline{H}_3$), 1.80 (dd, 3H, *J* = 6.9, 1.4 Hz, H-8), 1.27 (t, 3H, *J* = 7.2 Hz, H-3′); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 168.4 (C-1), 139.6 (C-5), 138.4 (C-3), 133.8 (C-7), 131.7 (C-6), 126.2 (C-2), 125.4 (C-4), 60.4 (C-2′), 18.5 (C-8), 14.3 (C-3′), 12.6 (2-$\underline{C}H_3$); HRMS (EI) calcd for $C_{11}H_{16}O_2$ 180.1150, found 180.1146 $[M]^+$.

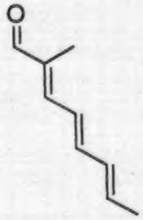
(2*E*,4*E*,6*E*)-2-Methylocta-2,4,6-trien-1-ol (35)



Neat DIBAL-H (3.94 mL, 22.1 mmol) was added dropwise to a cooled (-78 °C) stirred solution of **34** (1.8971 g, 10.5 mmol) in dry THF (50 mL) and the reaction mixture was allowed to stir and warm to rt over 5 h. The mixture was then cooled (-15 °C) and sat'd $NaHCO_3$ (30 mL) was added and the mixture was warmed to rt and filtered through a plug of silica (washing through with Et_2O (500 mL)). The filtrate was then washed with brine. The aqueous fraction was back extracted with Et_2O and the ethereal layers combined, dried ($MgSO_4$), filtered and the solvent evaporated to yield **35** (1.4027 g, 97%) as an oil. (SiO_2, 1:1/Et_2O:pentane, R_f 0.48). IR (microscope) 3409 (br), 2975, 2932, 1713, 1635, 1450, 1375, 1260, 1055 cm^{-1}; ^1H NMR ($CDCl_3$, 600 MHz) δ 6.30 (dd, 1H, *J* = 14.4, 10.8 Hz, H-4), 6.17 (dd, 1H, *J* = 14.4, 10.8 Hz, H-5), 6.15-6.06 (m, 1H, H-6), 6.04 (d, 1H, *J* = 10.8 Hz, H-3), 5.70 (dq, 1H, *J* = 14.4, 7.2 Hz, H-7), 4.06 (s, 2H, H-1), 1.78 (s, 3H, 2-C$\underline{H}_3$), 1.76, (d, 3H, *J* = 7.2 Hz, H-8); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 136.5 (C-2), 133.0 ($\underline{C}$H), 131.9 (C-5), 129.7

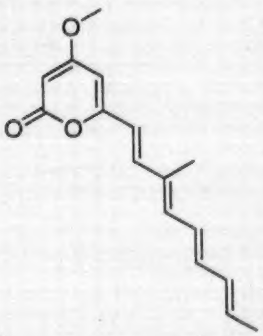
(CH), 125.9 (CH), 125.3 (C-3), 68.6 (C-1), 18.3 (C-8), 14.3 (2-CH$_3$); HRMS (EI) calcd for $C_9H_{15}O$ 138.1045, found 138.1047 [M]$^+$.

(2*E*,4*E*,6*E*)-2-Methylocta-2,4,6-trienal (36)



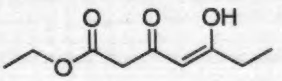
The known compound **36**[144] was prepared using the same method as for the preparation of **102**. Thus, reaction of **35** (115.4 mg, 835 μmol) in dry CH_2Cl_2 (2 mL) with DMP (425 mg, 1.0 mmol) in dry CH_2Cl_2 (10 mL) overnight to afforded **36** as a light yellow oil (112.8 mg, 99%) after purification by flash chromatography (SiO_2, 1:9/Et_2O:pentane) (SiO_2, 1:1/Et_2O:pentane, R_f 0.83). Storage under argon is important as the product polymerizes or decomposes to an insoluble solid after prolonged exposure to air, either at rt or -20 °C. IR (CH_2Cl_2 cast) 3026, 2917, 2851, 2818, 2713, 1678, 1639, 1611, 1436, 1406, 1377, 1359, 1240, 1194 cm^{-1}; ^{1}H NMR (CD_2Cl_2, 400 MHz) δ 9.41 (s, 1H, H-1), 6.86 (dq, 1H, *J* = 10.6, 1.2 Hz, H-3), 6.65 (dd, 1H, *J* = 14.8, 10.4 Hz, H-5), 6.56 (dd, 1H, *J* = 14.8, 10.6 Hz, H-4), 6.31-6.23 (m, 1H, H-6), 6.04 (dq, 1H, *J* = 14.0, 6.8 Hz, H-7), 1.85 (dd, 3H, *J* = 6.8, 1.2 Hz, H-8), 1.82 (d, 3H, *J* = 1.2 Hz, 2-CH$_3$); ^{13}C NMR (CD_2Cl_2, 100 MHz) δ 194.7 (C-1), 149.0 (C-3), 142.0 (C-5), 137.4 (C-2), 136.5 (C-7), 131.9 (C-6), 125.4 (C-4), 18.8 (C-8), 9.6 (2-CH$_3$); HRMS (EI) calcd for $C_9H_{12}O$ 136.0888, found 136.0885 [M]$^+$.

4-Methoxy-6-[(1*E*,3*E*,5*E*,7*E*)-3-methylnona-1,3,5,7-tetraen-1-yl]-2*H*-pyran-2-one (37)



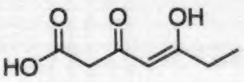
The same method as for the preparation of **31** was employed. *n*-BuLi (1.6 M in hexanes, 490 μL, 787 μmol) was added to a stirred suspension of the Wittig salt **29** (344 mg, 716 μmol) in dry THF (5 mL) at rt. The mixture was allowed to stir for 1 hr before a solution of the aldehyde **36** (88.6 mg, 1.9 mmol) in dry THF (3 mL) was added and stirred for 2 days. The solvent was then evaporated under reduced pressure and the mixture was purified by flash chromatography (SiO_2, 1:2/EtOAc, pentane) to yield **37** (71.6 mg, 42%) as a yellow solid. (SiO_2, 2:1/EtOAc, pentane, R_f 0.62). HPLC (Varian C_{18} column, MeCN and H_2O, 1mL/min, 220 & 280 nm), 30% MeCN to 50% MeCN over 12 min and staying there for 5 min before increasing to 70% over 6 min then increasing to 100% over 2 min and staying there for 5 min before decreasing to 30% and staying there for 5 min (t_r 29.19 min); IR (microscope) 3398 (br), 3087, 2920, 1720, 1636, 1551, 1454, 1409, 1253 cm^{-1}; ^1H NMR ($CDCl_3$, 600 MHz) δ 7.19 (d, 1H, *J* = 15.6 Hz, H-2′), 6.45-6.31 (m, 3H, H-4′ & H-5′ & H-6′), 6.17 (ddd, 1H, *J* = 15.0, 10.8, 1.8 Hz, H-7′), 5.99 (d, 1H, *J* = 15.6 Hz, H-1′), 5.87-5.77 (m, 1H, H-8′), 5.81 (d, 1H, *J* = 1.8 Hz, H-5), 5.42 (d, 1H, *J* = 1.8 Hz, H-3), 3.78 (s, 3H, $OC\underline{H}_3$), 1.89 (s, 3H, 3′-$C\underline{H}_3$), 1.80 (d, 3H, *J* = 7.5 Hz, H-9′); ^{13}C NMR ($CDCl_3$, 125 MHz) δ171.2 (C-4), 164.2 (C-2), 159.4 (C-6), 140.4 (C-2′), 137.6 (C-6′), 136.9 (C-4′), 133.1 (C-3′), 132.4 (C-7′), 132.0 (C-8′), 126.3 (C-5′), 116.9 (C-1′), 100.3 (C-5), 88.2 (C-3), 55.8 ($O\underline{C}H_3$), 18.5 (C-9′), 12.3 (3′-$\underline{C}H_3$); HRMS (EI) calcd for $C_{16}H_{18}O_3$ 258.1256, found 258.1258 $[M]^+$.

Ethyl 3,5-dioxoheptanoate (39)



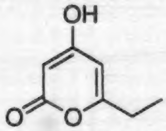
The known compound **39**[145] was prepared using a modification of the method of Huckin and Weiler.[120] Ethyl acetoacetate (489 μL, 3.84 mmol) was added dropwise to a cooled (0 °C) stirred solution of NaH (60% in mineral oil) (154 mg, 3.84 mmol) in dry THF (10 mL) and the reaction mixture stirred for 10 min. *n*-BuLi (1.6 M in hexanes, 2.4 mL, 3.84 mmol) was added and the reaction mixture stirred for 10 min. To this mixture was added ethyl propionate (220 μL, 1.92 mmol) and the reaction mixture stirred for 15 min. *n*-BuLi (1.6 M in hexanes, 2.4 mL, 3.84 mmol) was added and the mixture stirred for 10 min. To this mixture was added ethyl propionate (220 μL, 1.92 mmol) and the reaction mixture stirred for 30 min before it was acidified with conc. HCl (1 mL) (reaction turned from orange to a pale yellow, and generated heat), H_2O (5 mL) and Et_2O (15 mL). The layers were separated and the aqueous layer extracted with Et_2O (twice). The ethereal layers were combined, washed with brine, dried (Na_2SO_4), filtered and the solvent evaporated under reduced pressure. The purification of the mixture was difficult since the product and ethyl acetoacetate have nearly the same R_f and chromatographic properties, but purification was accomplished by flash chromatography (SiO_2, 1:4/Et_2O:pentane) to yield **39** (166.9 mg, 23%) (SiO_2, 1:1/Et_2O:pentane, R_f 0.67). Product appears to prefer the enol form by ^{1}H-NMR. IR (microscope) 2981, 2941, 1740, 1606 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.57 (s, 1H, H-4), 4.16 (q, 2H, J = 7.2 Hz, H-2′), 3.29 (s, 2H, H-2), 2.30 (q, 2H, J = 7.6 Hz, H-6), 1.24 (t, 3H, J = 7.2 Hz, H-3′), 1.10 (t, 3H, J = 7.6 Hz, H-7); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 194.5 (C-5), 186.7 (C-3), 167.6 (C-1), 99.1 (C-4), 61.4 (C-2′), 45.0 (C-2), 31.0 (C-6), 14.0 (C-3′), 9.5 (C-7); HRMS (EI) calcd for $C_9H_{14}0_4$ 186.0892, found 186.0894 $[M]^+$.

3,5-Dioxoheptanoic acid (40)



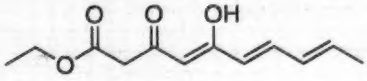
The known compound **40**[146] was prepared as follows. LiOH (1 M, 118 μL) was added to a stirred solution of **39** (20.2 mg, 107 μmol) in EtOH (1 mL) and stirred for 4 h. The mixture was then diluted witih H_2O and Et_2O, the layers separated and the organic layer extracted with H_2O. The aqueous layers were combined and acidified (pH 2) with 4 N HCl, and extracted with Et_2O (twice). The ethereal layers were combined, dried (Na_2SO_4), filtered and the solvent evaporated to yield **40** (8 mg, 47%) which was carried on to the next reaction without further purification. IR (microscope) 3400-3000 (br), 2958, 2927, 2855, 1708, 1659, 1613 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 5.59 (s, 1H, H-4), 3.40 (s, 2H, H-2), 2.34 (q, 2H, *J* = 7.6 Hz, H-6), 1.14 (t, 3H, *J* = 7.6 Hz, H-7); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 99.1 (C-4), 44.1 (C-2), 30.6 (C-6), 9.7 (C-7), quaternary carbons are not seen due to the small amount of product; HRMS (EI) calcd for $C_7H_{10}0_4$ 158.0579, found 158.0576 $[M]^+$.

6-Ethyl-4-hydroxy-2*H*-pyran-2-one (41)



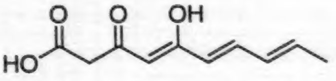
The known compound **41**[147] was prepared using a modification of the method of Evans and Staunton.[119] *n*-BuLi (1.6 M in hexanes, 990 μL, 1.59 mmol) and 2 drops of HMPT were added to a cooled (-78 °C) stirred solution of 4-hydroxy-6-methyl-2-pyrone (**20**) (100 mg, 790 μmol) in dry THF (5 mL) and the reaction mixture was left to stir and warm to rt for 2 h, at which point it turned deep red. MeI (100 μL, 1.59 mmol) was added and the reaction mixture stirred for 15 h before adding ice water. The solution was acidified (pH 2-3) with HCl and extracted with Et_2O (thrice). The organic fractions were combined, dried (Na_2SO_4), filtered and the solvent evaporated under reduced pressure. The mixture was difficult to separate due to the similarity of the starting material pyrone to the product, but separation was accomplished by flash chromatography (SiO_2, 2:1/EtOAc:pentane, R_f 0.17) to yield **41** (23.1 mg, 21%) as a white solid. IR (microscope) 3200-2500 (br), 3085, 2977, 2929, 2638, 1693, 1664, 1572, 1444, 1242 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 5.96 (d, 1H, *J* = 2.1 Hz, H-5), 5.55 (d, 1H, *J* = 2.1 Hz, H-3), 2.51 (q, 2H, *J* = 7.5 Hz, H-1′), 1.21 (t, 3H, *J* =7.5 Hz, H-2′); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 172.7 (C-4), 168.4 (C-2), 168.3 (C-6), 100.5 (C-5), 89.8 (C-3), 26.8 (C-1′), 10.8 (C-2′); HRMS (EI) calcd for $C_7H_8O_3$ 140.0473, found 140.0465 $[M]^+$.

Ethyl (4*Z*,6*E*,8*E*)-5-hydroxy-3-oxodeca-4,6,8-trienoate (43)



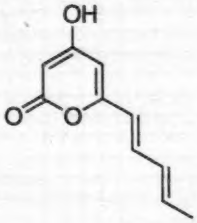
The same method, amounts and times as for the preparation of **39** was employed. Thus, reaction of ethyl acetoacetate (500 mg, 384 mmol) with ethyl sorbate (**42**) (2 X 281 μL) afforded **43** (202.6 mg, 23%) after purification by flash chromatography (SiO_2, 1:4/Et_2O:pentane) (SiO_2, 1:1/Et_2O:pentane, R_f 0.65). IR (microscope) 3457 (br), 2982, 2936, 1737, 1656, 1585 cm^{-1}; ^{1}H NMR ($CDCl_3$, 600 MHz) δ 7.18 (dd, 1H, *J* = 15.6, 10.8 Hz, H-7), 6.21-6.07 (m, 2H, H-8 & H-9), 5.79 (dd, 1H, *J* = 15.6, 0.6 Hz, H-6), 5.59 (s, 1H, H-4), 4.18 (q, 2H, *J* = 7.2 Hz, H-2′), 3.37 (s, 2H, H-3), 1.84 (dd, 3H, *J* = 6.0, 0.6 Hz, H-10), 1.26 (t, 3H, *J* = 7.2 Hz, H-3′); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 192.3 (C-3), 177.0 (C-5), 167.8 (C-1), 141.2 (C-7), 139.2 (C-9), 130.5 (C-8), 123.2 (C-6), 100.1 (C-4), 61.4 (C-2′), 46.8 (C-2), 18.8 (C-10), 14.1 (C-3′); HRMS (EI) calcd for $C_{12}H_{16}O_4$ 224.1049, found 224.1050 $[M]^+$.

(4*Z*,6*E*,8*E*)-5-Hydroxy-3-oxodeca-4,6,8-trienoic acid (44)



The same method as for the preparation of **40** was employed. Thus, reaction of **43** (65 mg, 290 μmol) with LiOH (1 M, 318 μL) in EtOH (5 mL) afforded **44** (40.7 mg, 72%). Which was carried on without further purification. IR (microscope) 3550-3000 (br), 2960, 2932, 1729, 1656, 1592 cm^{-1}; ^{1}H NMR (CDCl$_3$, 400 MHz) δ 10.40 (br s, COOH), 7.24 (dd, 1H, *J* = 15.2, 9.6 Hz, H-7), 6.25-6.10 (m, 2H, H-8 & H-9), 5.79 (d, 1H, *J* = 15.2 Hz, H-6), 5.58 (s, 1H, H-4), 3.45 (s, 2H, H-3), 1.86 (d, 3H, *J* = 5.2 Hz, H-10); ^{13}C NMR (CDCl$_3$, 100 MHz) δ 193.0 (C-3), 177.6 (C-5), 171.1 (C-1), 142.3 (C-7), 140.2 (C-9), 130.4 (C-8), 122.6 (C-6), 100.1 (C-4), 45.1 (C-2), 18.8 (C-10); HRMS (EI) calcd for $C_{10}H_{12}O_4$ 196.0736, found 196.0740 [M]$^+$.

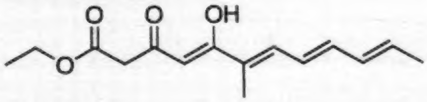
4-Hydroxy-6-[(1*E*,3*E*)-penta-1,3-dien-1-yl]-2*H*-pyran-2-one (45)



The same method as for the preparation of **3** was employed. Thus, reaction of the acid **44** (37.2 mg, 189 μmol) with TFAA (230.3 μL) and TFA (120 μL) at 70 °C for 1 hr afforded **45** (10.5 mg, 31%) after purification by flash chromatography (SiO$_2$, 1:1/Et$_2$O:pentane, flushing with 10% MeOH in EtOAc to elute product) and crystallization with EtOAc. The H-3 proton exchanges quickly in CD$_3$OD but can be replaced when dried and resuspended in CH$_3$OH. IR (microscope) 3200-2800 (br), 3040, 2997, 2964, 2946, 2913, 1701, 1626, 1598, 1561, 1445 cm^{-1}; ^{1}H NMR (CD$_3$OD, 500 MHz) δ 7.04 (dd, 1H, *J* = 15.5, 11.0 Hz, H-2′), 6.29-6.21 (m, 1H, C-3′), 6.14-6.05 (m, 2H, H-1′ & H-4′), 6.02 (s, 1H, H-5), 5.35 (d, 1H, *J* = 1.6 Hz, H-3), 1.84 (dd, 3H, *J* = 6.5, 1.0 Hz, H-5′); ^{13}C NMR (CD$_3$OD, 125

MHz) δ 173.2 (C-4), 167.5 (C-2), 161.7 (C-6), 137.9 (CH), 137.6 (CH), 131.8 (CH), 121.2 (CH), 102.0 (C-5), 90.6 (C-3), 18.7 (C-5′); HRMS (EI) calcd for $C_{10}H_{10}O_3$ 178.0630, found 178.0630 $[M]^+$.

Ethyl (4*Z*,6*E*,8*E*,10*E*)-5-hydroxy-6-methyl-3-oxododeca-4,6,8,10-tetraenoate (46) (Method a)

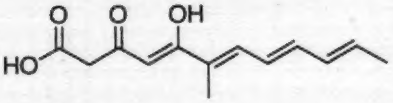


The same method, and times as for the preparation of **39** was employed. Thus, reaction of ethyl acetoacetate (212 μL, 1.66 mmol) with **34** (2 X (150 mg in 1 mL THF)), NaH (60% in mineral oil, 66.5 mg, 1.66 mmol) and *n*-BuLi (1.6 M in hexanes, 2 X (1.04 mL, 1.66 mmol)) afforded **46** (202.9 mg, 46%) after purification by flash chromatography (SiO_2, 1:4/Et_2O:pentane), (SiO_2, 1:1/Et_2O:pentane, R_f 0.64). IR (CH_2Cl_2 cast) 2982, 2934, 1737, 1591, 1442, 1255 cm^{-1}; UV (CH_2Cl_2 solution) λ_{max} 359, 207 nm; ^1H NMR ($CDCl_3$, 600 MHz) δ 7.16 (d, 1H, *J* = 11.4 Hz, H-7), 6.51 (dd, 1H, *J* =15.0, 10.8 Hz, H-9), 6.41 (dd, 1H, *J* = 15.0, 11.4 Hz, H-8), 6.19 (ddq, 1H, *J* = 15.0, 10.8, 1.2 Hz, H-10), 5.91 (dq, 1H, *J* = 15.0, 7.2 Hz, H-11), 5.85 (s, 1H, H-4), 4.18 (q, 2H, *J* = 7.2 Hz, H-2′), 3.38 (s, 2H, H-2), 1.89 (s, 3H, 6-CH$_3$), 1.81 (dd, 3H, *J* = 7.2, 1.2 Hz, H-12), 1.26 (t, 3H, *J* = 7.2 Hz, H-3′); ^{13}C NMR ($CDCl_3$, 150 MHz) δ 190.0 (C-3), 181.8 (C-5), 168.0 (C-1), 140.3 (C-9), 136.7 (C-7), 134.7 (C-11), 131.6 (C-10), 130.0 (C-6), 125.6 (C-8), 96.6 (C-4), 61.3 (C-2′), 46.2 (C-2), 19.2 (C-12), 14.0 (C-3′), 12.1 (6-CH$_3$); HRMS (EI) calcd for $C_{15}H_{20}O_4$ 264.1362, found 264.1365 $[M]^+$.

Ethyl (4Z,6*E*,8*E*,10*E*)-5-hydroxy-6-methyl-3-oxododeca-4,6,8,10-tetraenoate (46) (Method b)

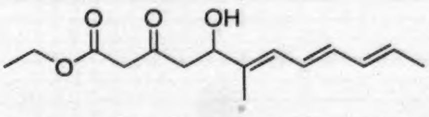
The same method as for the preparation of **102** was employed. Thus, reaction of **48** (7.5 mg, 28.1 µmol) in dry CH_2Cl_2 (1 mL) with DMP (14.3 mg, 34 µmol) in dry CH_2Cl_2 (1 mL) overnight afforded **46** (3.6 mg, 48%) after purification by flash chromatography (SiO_2, 1:1/Et_2O:pentane). The product had the same spectroscopic properties as **46** prepared by Method a.

(4Z,6*E*,8*E*,10*E*)-5-Hydroxy-6-methyl-3-oxododeca-4,6,8,10-tetraenoic acid (47)



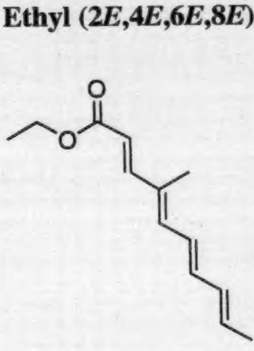
The same method as for the preparation of **40** was employed. Thus, reaction of **46** (93 mg, 352 µmol) with LiOH (1 M, 387 µL) in EtOH (4 mL) afforded **47** (68 mg, 81%). Which was carried on without further purification. IR (CH_2Cl_2 cast) 3300-2700 (br), 2934, 1719, 1593, 1557, 1435, 1250, 988 cm^{-1}; UV (CH_2Cl_2 solution) λ_{max} 380, 207 nm; ^{1}H NMR ($CDCl_3$, 600 MHz) δ 9.60 (br s, COO$\underline{\text{H}}$), 7.21 (d, 1H, *J* = 12.0 Hz, H-7), 6.55 (dd, 1H, *J* =14.7, 10.8 Hz, H-9), 6.41 (dd, 1H, *J* = 14.7, 12.0 Hz, H-8), 6.20 (ddq, 1H, *J* = 15.0, 10.8, 1.5 Hz, H-10), 5.94 (dq, 1H, *J* = 15.0, 6.6 Hz, H-11), 5.81 (s, 1H, H-4), 3.48 (s, 2H, H-2), 1.89 (s, 3H, 6-C$\underline{\text{H}}_3$), 1.81 (dd, 3H, *J* = 6.6, 1.5 Hz, H-12); ^{13}C NMR ($CDCl_3$, 150 MHz) δ 191.2 (C-3), 181.5 (C-5), 171.2 (C-1), 141.5 (C-9), 137.7 (C-7), 135.2 (C-11), 131.4 (C-10), 128.5 (C-6), 125.7 (C-8), 96.5 (C-4), 45.2 (C-2), 19.0 (C-12), 12.2 (6-$\underline{\text{C}}$H$_3$); HRMS (EI) calcd for $C_{13}H_{16}O_4$ 236.1049, found 236.1041 [M]$^+$.

Ethyl (6*E*,8*E*,10*E*)-5-hydroxy-6-methyl-3-oxododeca-6,8,10-trienoate (48)



A modification of the method of Trauner and coworkers was used.[121] A solution of ethyl acetoacetate (46.8 μL, 367 μmol) in dry THF (1 ML) was added dropwise to a cooled (0 °C) stirred solution of NaH (60% in mineral oil) (16.2 mg, 404 μmol) in dry THF (0.5 mL). The reaction mixture was stirred for 10 min before *n*-BuLi (1.6 M in hexanes, 252 μL, 404 μmol) was added and stirred for 10 min. To this mixture was added the aldehyde **36** (50 mL, 357 μmol) in dry THF (1 mL) and the reaction mixture stirred and warmed to rt for 6 h. The mixture was diluted with H_2O, Et_2O and acidified with conc. HCl (0.5 mL). The layers were separated and the aqueous layer extracted with Et_2O (twice). The ethereal layers were combined, dried (Na_2SO_4), filtered and the solvent evaporated under reduced pressure. The mixture was purified by flash chromatography (SiO_2, 1:4/Et_2O:pentane and washed with 1:1/Et_2O:pentane to elute the crude product) to yield **48** (7.5 mg, 8%) after a second column (SiO_2, 1:1/Et_2O:pentane, R_f 0.42). IR (microscope) 3324, 3026, 2931, 2819, 2714, 1678, 1639, 1610 cm^{-1}; ^{1}H NMR ($CDCl_3$, 600 MHz) δ 6.25 (dd, 1H, *J* = 15.0, 10.2 Hz, H-8), 6.17 (dd, 1H, *J* = 14.4, 10.2 Hz, H-9), 6.14-6.05 (m, 2H, H-7 & H-10), 5.70 (dq, 1H, *J* = 14.4, 7.2 Hz, H-11), 4.53 (dd, 1H, *J* = 9.0, 3.0 Hz, H-5), 4.38 (q, 2H, *J* = 7.2 Hz, H-2′), 3.47 (s, 2H, H-2), 2.79 (dd, 1H, *J* = 17.0, 9.0 Hz, 1 X H-4), 2.61 (dd, 1H, *J* = 17.0, 3.0 Hz, 1 X H-4), 1.76 (d, 3H, *J* = 7.2 Hz, H-12), 1.74 (s, 3H, 6-C$\underline{H}_3$), 1.26 (t, 3H, *J* = 7.2 Hz, H-3′); ^{13}C NMR ($CDCl_3$, 150 MHz) δ 202.6 (C-3), 166.5 (C-1), 137.0 (C-6), 133.7 (C-9), 130.1 (C-11), 126.3 (C-7), 125.8 (C-8), 125.7 (C-10), 72.5 (C-5), 61.7 (C-2′), 50.1 (C-2), 48.3 (C-4), 18.6 (C-12), 14.2 (C-3′), 13.1 (6-$\underline{C}H_3$); HRMS (EI) calcd for $C_{15}H_{22}0_4$ 266.1518, found 266.1518 [M]$^+$.

Ethyl (2*E*,4*E*,6*E*,8*E*)-4-methyldeca-2,4,6,8-tetraenoate (49) (Method a)

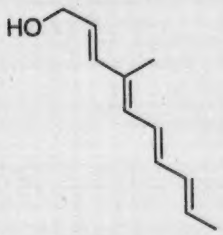


The same method as for the preparation of **34** was employed. Thus, reaction of **36** (300 mg, 2.2 mmol) in dry CH_2Cl_2 (2 mL) with (carbethoxymethylene)triphenylphosphorane (1.04 g, 2.4 mmol) in dry CH_2Cl_2 (18 mL) at rt overnight then heating to 35 °C for 3 days afforded the product **49** (312.3 mg, 69%) after purification by flash chromatography, 2 columns, (SiO_2, 5:95/Et_2O:pentane) (SiO_2, 1:4/Et_2O:pentane, R_f 0.59). IR (CH_2Cl_2 cast) 3025, 2980, 2933, 1708, 1619, 1589, 1298, 1172, 986 cm^{-1}; UV (CH_2Cl_2 solution) λ_{max} 335 nm; ^1H NMR ($CDCl_3$, 600 MHz) δ 7.32 (d, 1H, J = 15.6 Hz, H-3), 6.44-6.32 (m, 3H, H-5 & H-6 & H-7), 6.16 (ddq, 1H, J = 15.0, 10.2, 1.5 Hz, H-8), 5.88-5.79 (m, 2H, H-2 & H-9), 4.19 (q, 2H, J = 7.2 Hz, H-2′), 1.86 (s, 3H, 4-C$\underline{H}_3$), 1.79 (dd, 3H, J = 7.2, 1.5 Hz, H-10), 1.28 (t, 3H, J = 7.2 Hz, H-3′); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 167.4 (C-1), 148.9 (C-3), 138.8 (C-7), 137.5 (C-5), 132.8 (C-9), 132.7 (C-4), 131.9 (C-8), 126.0 (C-6), 116.2 (C-2), 60.1 (C-2′), 18.5 (C-10), 14.3 (C-3′), 12.4 (4-$\underline{C}H_3$); HRMS (EI) calcd for $C_{13}H_{18}O_2$ 206.1307, found 206.1304 $[M]^+$.

Ethyl (2*E*,4*E*,6*E*,8*E*)-4-methyldeca-2,4,6,8-tetraenoate (49) (Method b)

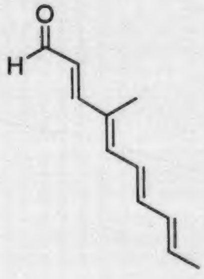
Et_3N (399 μL, 2.84 mmol) and LiBr (246 mg) were added to a cooled (0 °C) stirred solution of triethyl phosphonoacetate (636 mg, 2.84 mmol) in dry CH_2Cl_2 (20 mL) and reaction mixture stirred for 20 min. A solution of the aldehyde **36** (351.4 mg, 2.58 mmol) in dry CH_2Cl_2 (5 mL) was then added and the reaction mixture was stirred for 5 days. The reaction did not to go to completion by TLC. The solvent was evaporated and purified by flash chromatography (SiO_2, 1:4/Et_2O:pentane) followed by further purification by flash chromatography, 3 columns, (SiO_2, 1:9/Et_2O:pentane) to yield **49** (207 mg, 39 %) along with unreacted **36** (300 mg).

(2*E*,4*E*,6*E*,8*E*)-4-Methyldeca-2,4,6,8-tetraen-1-ol (50)



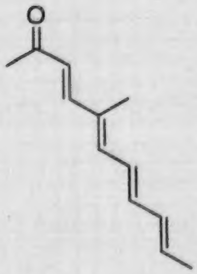
The same method as for the preparation of **35** was employed. Thus, reaction of **49** (253.2 mg, 1.23 mmol) in dry THF (5 mL) at -78 °C, with DIBAL-H (459 μL, 2.577 mmol) for 6 h afforded **50** (137.5 mg, 68%) after purification by flash chromatography (SiO_2, 1:1/Et_2O:pentane, R_f 0.25). The product decomposed quickly. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.38 (dd, 1H, *J* = 14.0, 11.2 Hz, H-6), 6.30-6.10 (m, 3H, H-3 & H-7 & H-8), 6.03 (d, 1H, *J* = 11.2 Hz, H-5), 5.85-5.65 (m, 2H, H-2 & H-9), 4.17 (t, 2H, *J* = 5.6 Hz, H-1), 1.83 (s, 3H, 4-C$\underline{H}_3$), 1.75 (d, 3H, *J* = 7.2 Hz, H-10); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 136.1 (C-3), 133.8 (C-7), 133.6 (C-4), 132.0 (C-5), 131.6 (C-9), 130.1 (C-8), 126.8 (C-2), 126.5 (C-6), 63.2 (C-1), 18.2 (C-10), 15.1 (4-$\underline{C}H_3$).

(2*E*,4*E*,6*E*,8*E*)-4-Methyldeca-2,4,6,8-tetraenal (51)



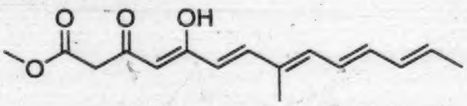
The same method as for the preparation of **36** was employed. Thus, reaction of **50** (137.5 mg, 837 μmol) in dry CH_2Cl_2 (5 mL) with DMP (426 mg, 1.0 mmol) in dry CH_2Cl_2 (5 mL) for 45 min afforded **51** (21.9 mg, 16%) as a yellow oil after purification by flash chromatography (SiO_2, 1:1/Et_2O:pentane, R_f 0.69). ^{1}H NMR (CD_2Cl_2, 400 MHz) δ 9.55 (d, 1H, *J* = 7.6 Hz, H-1), 7.15 (d, 1H, *J* = 15.2 Hz, H-3), 6.65-6.45 (m, 3H, H-5 & H-6 & H-7), 6.28-6.19 (m, 1H, H-8), 6.14 (dd, 1H, *J* = 15.2, 7.6 Hz, H-2), 5.95 (dq, 1H, J = 14.0, 7.0 Hz, H-9), 1.94 (s, 3H, 4-C$\underline{H}_3$), 1.83 (dd, 3H, J = 7.0, 1.6 Hz, H-10); ^{13}C NMR (CD_2Cl_2, 100 MHz) δ 193.8 (C-1), 156.9 (C-3), 141.0 ($\underline{C}$H), 139.4 ($\underline{C}$H), 134.4 ($\underline{C}$H), 133.6 (C-4), 132.2 ($\underline{C}$H), 127.5 ($\underline{C}$H), 126.4 ($\underline{C}$H), 18.7 (C-10), 12.8 (4-$\underline{C}H_3$); HRMS (EI) calcd for $C_{11}H_{14}O$ 162.1045, found 162.1040 $[M]^+$.

(3*E*,5*E*,7*E*,9*E*)-5-Methylundeca-3,5,7,9-tetraen-2-one (52)



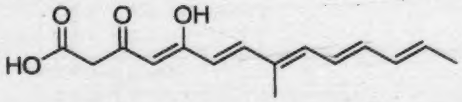
The same method as for the preparation of **34** was employed. Thus, reaction of **36** (300 mg, 2.2 mmol) in dry CH_2Cl_2 (2 mL) with acetonyltriphenylphosphorane (859 mg, 2.4 mmol) in dry CH_2Cl_2 (10 mL) at rt overnight then heating to 40 °C for 3 h and 30 °C for 3 days afforded the product **52** (202.6 mg, 52%) after purification by flash chromatography, (SiO_2, 1:4/Et_2O:pentane, R_f 0.27). IR (microscope) 3445 (br), 2986, 2919, 1710, 1666, 1639, 1584 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.18 (d, 1H, J = 15.8 Hz, H-4), 6.50-6.38 (m, 3H, H-6 & H-7 & H-9), 6.26-6.12 (m, 1H, H-8), 6.13 (d, 1H, J = 15.8 Hz, H-3), 5.89 (dq, 1H, J = 13.9, 6.9 Hz, H-10), 2.25 (s, 3H, H-1), 1.90 (s, 3H, 5-C$\underline{H}_3$), 1.82 (dd, 3H, J = 6.9, 1.4 Hz, H-11); ^{13}C NMR (CD_2Cl_2, 125 MHz) δ 198.3 (C-2), 147.8 (C-4), 140.0 (C-6), 138.2 (C-9), 133.6 (C-5), 133.6 (C-10), 132.3 (C-8), 126.6 (C-7), 126.0 (C-3), 27.6 (C-1), 18.7 (C-11), 12.7 (5-$\underline{C}H_3$); HRMS (EI) calcd for $C_{12}H_{16}O$ 176.1201, found 176.1201 $[M]^+$.

Methyl (4*Z*,6*E*,8*E*,10*E*,12*E*)-5-hydroxy-8-methyl-3-oxotetradeca-4,6,8,10,12-pentaenoate (53)



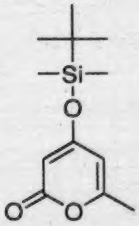
Diisopropylamine (87.5 μL, 624 μmol) and *n*-BuLi (1.6 M in hexanes, 429.4 μL, 687 μmol) were stirred together at -20 °C for 20 min. The mixture was added to a cooled (-78 °C) stirred solution of the ketone **52** (100 mg, 567 μmol) in dry THF (1 mL). Acetoxyacetyl chloride (66.9 μL, 624 μmol) was added to the reaction and it was left to stir and warm to rt over 5 h. The reaction mixture was diluted with Et_2O and H_2O and acidified (pH 2) with 2 N HCl. The layers were separated and the aqueous fraction extracted with Et_2O (five times). The organic fractions were combined, washed with brine, dried (Na_2SO_4), filtered and the solvent evaporated. The mixture was purified by flash chromatography (SiO_2, 1:1/Et_2O:pentane, R_f 0.46) to yield **53** (15.92 mg, 10%). IR (CH_2Cl_2 cast) 3430 (br), 2955, 2930, 2871, 1743, 1597, 1437, 1267, 1158, 973 cm^{-1}; UV (CH_2Cl_2 solution) λ_{max} 335, 281, 219 nm; ^1H NMR (CD_2Cl_2, 600 MHz) δ 7.31 (d, 1H, *J* = 15.6 Hz, H-7), 6.54-6.38 (m, 1H, H-11), 6.32-6.05 (m, 2H, H-10 & H-12), 5.96 (d, 1H, *J* = 15.6, H-6), 5.94-5.86 (m, 1H, H-9), 5.78-5.68 (m, 1H, H-13), 5.67 (s, 1H, H-4), 3.72 (s, 3H, H-2′), 3.40 (s, 2H, H-2), 1.92 (s, 3H, 8-$C\underline{H}_3$), 1.83 (dd, 3H, *J* = 7.2, 1.2 Hz, H-14); ^{13}C NMR (CD_2Cl_2, 150 MHz) δ 192.4 (C-3), 177.5 (C-5), 168.0 (C-1), 145.0 (C-7), 140.0 (C-8), 139.0 (C-11), 134.9 (C-13), 133.7 (C-9), 132.1 (C-12), 125.5 (C-10), 120.9 (C-6), 100.5 (C-5), 52.6 (C-2′), 47.0 (C-2), 19.2 (C-14), 12.4 (8-$\underline{C}H_3$); HRMS (EI) calcd for $C_{16}H_{20}O_4$ 276.1362, found 276.1361 $[M]^+$.

(4*Z*,6*E*,8*E*,10*E*,12*E*)-5-Hydroxy-8-methyl-3-oxotetradeca-4,6,8,10,12-pentaenoic acid (54)



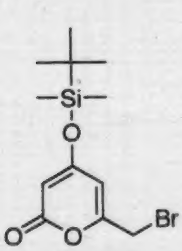
The same method as for the preparation of **40** was employed. Thus, reaction of **53** (7.95 mg, 28.7 μmol) with LiOH (1 M, 90 μL) in MeOH (0.5 mL) for 3 h afforded **54** (5.01 mg, 66%). Which was carried on without further purification. IR (CH_2Cl_2 cast) 3500-2500 (br), 2926, 1725, 1621, 1580, 1433 cm^{-1}; UV (CH_2Cl_2 solution) λ_{max} 385, 284 nm; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.36 (d, 1H, *J* = 15.5 Hz, H-7), 6.49-6.38 (m, 3H, H-9 & H-11 & H-12), 6.25-6.12 (m, 1H, H-10), 5.88 (d, 1H, *J* = 15.5, H-6), 5.92-5.82 (m, 1H, H-13), 5.59 (s, 1H, H-4), 3.48 (s, 2H, H-2), 1.89 (s, 3H, 8-C$\underline{H}_3$), 1.82 (dd, 3H, *J* = 7.0, 1.5 Hz, H-14); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 191.5 (C-3), 179.8 (C-5), 171.0 (C-1), 146.8 (C-7), 140.7 (C-8), 138.7 (C-11), 134.3 (C-13), 133.7 (C-9), 131.9 (C-12), 126.2 (C-10), 119.2 (C-6), 100.1 (C-5), 44.0 (C-2), 18.6 (C-14), 12.4 (8-$\underline{C}H_3$); LRMS (ES) calcd for $C_{15}H_{18}O_4$ 262.1, found 263.1 $[M+H]^+$, 285.1 $[M+Na]^+$, 547 $[2M+Na]^+$.

4-(*Tert*-butyldimethylsilyloxy)-6-methyl-2*H*-pyran-2-one (55)



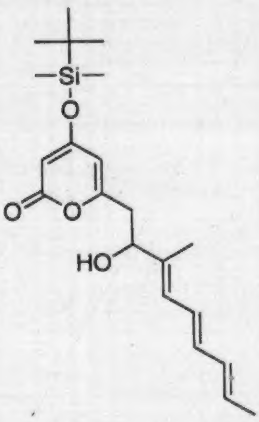
Pyridine (5.23 mL, 63,4 mmol) was added dropwise to a stirred suspension of 4-hydroxy-6-methyl-2-pyrone (**20**) (2 g, 15.86 mmol) in dry CH_2Cl_2 (20 mL). After 20 min, the solution became clear and a solution of TBDMS-Cl (3.343 g, 17.44 mmol) in dry CH_2Cl_2 (5 mL) was added dropwise and the reaction mixture stirred at rt for 40 h. The mixture was then washed with 2 N HCl (twice) and brine, dried ($MgSO_4$), filtered and the solvent evaporated under reduced pressure. The ^{1}H-NMR showed mostly product with some starting silyl derived impurity, possibly silyl chloride. The mixture was put under high vac at 35 °C overnight to yield pure product (3.6117 g, 95%) as an oil, which solidified to a white wax upon freezing. IR (microscope) 3200-2100 (br), 3097, 2926, 2820, 2726, 2621, 1754, 1672, 1627, 1586, 1541, 1494, 1306, 1257 cm^{-1}; ^{1}H NMR ($CDCl_3$, 600 MHz) δ 5.69 (d, 1H, *J* = 1.8 Hz, H-5), 5.37 (d, 1H, *J* = 1.8 Hz, H-3), 2.18 (s, 3H, H-1′), 0.93 (s, 9H, $C(C\underline{H}_3)_3$), 0.25 (s, 6H, $Si(\underline{C}H_3)_2$); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 168.5 (C-4), 164.8 (C-2), 162.5 (C-6), 102.9 (C-5), 94.4 (C-3), 25.0 ($C(\underline{C}H_3)_3$), 19.5 (C-1′), 17.7 ($\underline{C}(CH_3)_3$), -4.9 ($Si(\underline{C}H_3)_2$); HRMS (EI) calcd for $C_{12}H_{20}O_3Si$ 240.1182, found 240.1180 $[M]^+$.

6-(Bromomethyl)-4-(*tert*-butyldimethylsilyloxy)-2*H*-pyran-2-one (56)



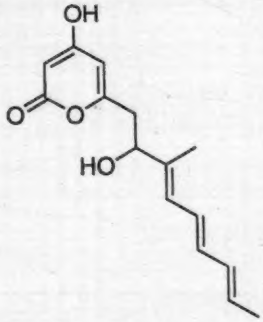
The same method as for the preparation of **28** was employed. Thus, reaction of **55** (1.6961 g, 7.05 mmol) with AIBN (116 mg, 705 μmol) and NBS (1.382 g, 7.76 mmol) in dry CCl_4 (50 mL) at (80 °C) for 22 h afforded **56** (2.5104 g), which was carried on without further purification. IR (microscope) 3300-2500 (br), 3085, 2752, 2643, 1775, 1698, 1669, 1565 cm^{-1}; ^{1}H NMR ($CDCl_3$, 600 MHz) δ 6.01 (d, 1H, *J* = 1.8 Hz, H-5), 5.47 (d, 1H, *J* = 1.8 Hz, H-3), 4.09 (s, 2H, H-1′), 0.95 (s, 9H, $C(C\underline{H}_3)_3$), 0.28 (s, 6H, $Si(\underline{C}H_3)_2$).

4-(*Tert*-butyldimethylsilyloxy)-6-[(3*E*,5*E*,7*E*)-2-hydroxy-3-methylnona-3,5,7-trien-1-yl]-2*H*-pyran-2-one (57)



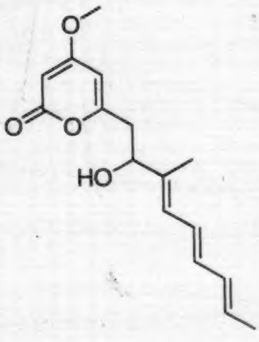
A modification of the method of Oikawa *et al* was used.[122] *n*-BuLi (1.6 M in hexanes, 626 μL, 1.00 mmol) was added to a cooled (-78 °C) stirred solution of **55** (219 mg, 911 μmol) in dry THF (2 mL). This mixture turned deep red and was allowed to stir for 10 min. A solution of the aldehyde **36** (112.8 mg, 828 μmol) in dry THF (2 mL) was added and the reaction mixture stirred for 3.5 h. A sat'd solution of NH_4Cl was added and the reaction mixture was allowed to warm to rt before extracting it with EtOAc. The aqueous fraction was back extracted with EtOAc, the organic fractions were combined, dried (Na_2SO_4), filtered and the solvent evaporated under reduced pressure. The mixture was then purified by flash chromatography (SiO_2, 5:95/acetone:chloroform, R_f 0.23) to yield **57** (6.9 mg, 2%). ^{1}H NMR ($CDCl_3$, 600 MHz) δ 6.25 (dd, 1H, *J* = 14.4, 10.8 Hz, H-5′), 6.18 (dd, 1H, *J* = 14.4, 10.2 Hz, H-6′), 6.13-6.04 (m, 2H, H-4′ & H-7′), 5.80 (d, 1H, *J* = 1.8 Hz, H-5), 5.72 (dq, 1H, *J* = 13.8, 6.6 Hz, H-8′), 5.40 (d, 1H, *J* = 1.8 Hz, H-3), 4.52-4.48 (m, 1H, H-2′), 2.63 (dd, 2H, *J* = 9.0, 4.2 Hz, H-1′), 1.78-1.75 (m, 6H, H-9 & 3-C$\underline{H}_3$), 0.94 (s, 9H, C(C$\underline{H}_3$)$_3$), 0.28 (s, 6H, Si($\underline{C}H_3$)$_2$); HRMS (EI) calcd for $C_{21}H_{32}O_4Si$ 376.2070, found 376.2068 $[M]^+$.

4-Hydroxy-6-[(3*E*,5*E*,7*E*)-2-hydroxy-3-methylnona-3,5,7-trien-1-yl]-2*H*-pyran-2-one (58)



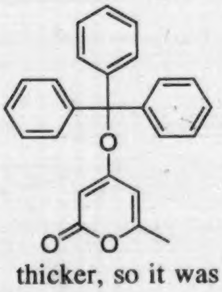
The same method as for the preparation of **57** was employed. Thus reaction of **55** (527 mg, 2.19 mmol) with *n*-BuLi (1.6 M in hexanes, 1.5 mL, 2.4 mmol) and the aldehyde **36** (271.3 mg, 1.99 mmol) in dry THF (10 mL) for 4 h at -78 °C did not afford **57** after work up and purification by flash chromatography (SiO_2, 2:1/EtOAc:pentane). Instead **58** (11.51 mg, 1.5%) was isolated (SiO_2, 2:1/EtOAc:pentane, R_f 0.11). IR (microscope) 2958, 2928, 2859, 1728, 1273 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 6.27-6.00 (m, 4H, H-4′ & H-5′ & H-6′ & H-7′), 6.05 (s, 1H, H-5), 5.70 (dq, 1H, *J* = 12.9, 6.6 Hz, H-8′), 5.49 (s, 1H, H-3), 4.50-4.42 (m, 1H, H-2′), 2.72-2.60 (m, 2H, H-1′), 1.78-1.75 (m, 6H, H-9 & 3-C$\underline{H}_3$); HRMS (EI) calcd for $C_{15}H_{18}O_4$ 262.1205, found 262.1204 $[M]^+$.

6-[(3*E*,5*E*,7*E*)-2-Hydroxy-3-methylnona-3,5,7-trien-1-yl]-4-methoxy-2*H*-pyran-2-one (59)



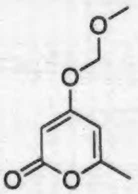
The same method as for the preparation of **57** was employed. Thus reaction of **27** (210 mg, 1.49 mmol) with *n*-BuLi (1.6 M in hexanes, 1.0 mL, 1.65 mmol) and the aldehyde **36** (204 mg, 1.49 mmol) in dry THF (5 mL) for 75 min at -78 °C afforded **59** (47.5 mg, 11%) after work up and purification by flash chromatography (SiO_2, 2:1/EtOAc:pentane, R_f 0.41). IR (microscope) 3404 (br), 3094, 2980, 2944, 1705, 1648, 1567, 1460, 1409, 1251 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 6.21 (dd, 1H, *J* = 14.2, 10.9 Hz, H-5′), 6.18-6.02 (m, 3H, H-4′ & H-6′ & H-7′), 5.80 (d, 1H, *J* = 2.3 Hz, H-5), 5.72 (dq, 1H, *J* = 14.0, 6.9 Hz, H-8′), 5.40 (d, 1H, *J* = 2.3 Hz, H-3), 4.52-4.48 (dd, 1H, *J* = 8.0, 5.2 Hz, H-2′), 3.73 (s, 3H, OCH_3), 2.63 (dd, 2H, *J* = 5.2, 2.3 Hz, H-1′), 1.78-1.70 (m, 6H, H-9′ & 3-CH_3); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 171.2 (C-4), 164.9 (C-2), 162.3 (C-6), 137.3 (C-3′), 133.8 (C-5′), 131.8 (C-7′), 130.2 (C-8′), 126.2 (C-4′), 125.5 (C-6′), 101.7 (C-5), 87.8 (C-3), 74.0 (C-2′), 55.8 (OCH_3), 40.0 (C-1′), 19.8 (C-9′), 12.3 (3′-CH_3); HRMS (EI) calcd for $C_{16}H_{20}O_4$ 276.1362, found 276.1358 $[M]^+$.

6-Methyl-4- (trityloxy)-2*H*-pyran-2-one (60)



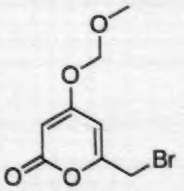
Et_3N (2.43 mL, 17.4 mmol) was added dropwise to a stirred suspension of the 4-hydroxy-6-methyl-2-pyrone (**20**) (2 g, 15.8 mmol) in dry CH_2Cl_2 (25 mL) and the mixture stirred for 10 min at rt before trityl chloride (4.863 g, 17.4 mmol) was added. The mixture became thicker, so it was diluted with dry CH_2Cl_2 (10 mL) and left to stir for 40 h. It was then diluted with H_2O and the layers separated. The aqueous fraction was back extracted with dry CH_2Cl_2 and the organic fractions combined, dried (Na_2SO_4), filtered and the solvent evaporated under reduced pressure to yield **60** (2.7529 g, 47%) as crystals after recrysallization with EtOAc. IR (microscope) 3083, 3054, 3030, 1722, 1664, 1613, 1542, 1492, 1446, 1399, 1355 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 7.47-7.40 (m, 6H, Ar-H), 7.30-7.22 (m, 6H, Ar-H), 7.20-7.15 (m, 3H, Ar-H), 6.36 (s, 1H, H-3), 5.70 (q, 1H, *J* = 1.0 Hz, H-5), 2.16 (d, 3H, *J* = 1.0 Hz, H-1′); ^{13}C NMR (CD_2Cl_2, 125 MHz) δ 166.3 (C-4), 164.0 (C-2), 161.7 (C-6), 145.2 (Ar-C), 129.6 (Ar-CH), 128.2 (Ar-CH), 126.5 (Ar-CH), 107.6, (C-3), 102.0 (C-5), 60.4 (OC(Ph)$_3$), 19.7 (C-1′), 14 carbons not seen due to symmetry; HRMS (ES) calcd for $C_{25}H_{20}O_3$ 368.1412, found 369.1484 $[M+H]^+$.

4-(Methoxymethoxy)-6-methyl-2*H*-pyran-2-one (61)



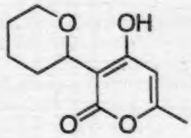
The same method as for the preparation of **60** was employed to make the known compound **61**.[148] Thus, reaction of 4-hydroxy-6-methyl-2-pyrone (**20**) (2 g, 15.8 mmol) with Et_3N (2.43 mL, 17.4 mmol) and MOM chloride (1.32 mL, 17.4 mmol) in dry CH_2Cl_2 (25 mL) for 40 h afforded **61** (2.2583 g, 84%) after purification by flash chromatography (SiO_2, 2:1/EtOAc:pentane, R_f 0.51). IR (microscope) 3092, 2961, 2835, 1727, 1650, 1567, 1453 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 5.79-5.77 (m, 1H, H-5), 5.55 (d, 1H, *J* = 2.4 Hz, H-3), 5.10 (s, 2H, OCH$_2$O), 3.43 (s, 3H, OCH$_3$), 2.17 (d, 3H, *J* = 0.4 Hz, H-1′); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 168.7 (C-4), 164.6 (C-2), 162.6 (C-6), 100.1 (C-5), 94.2 (OCH$_2$O), 90.2 (C-3), 56.9 (OCH$_3$), 19.9 (C-1′); HRMS (EI) calcd for $C_8H_{10}O_4$ 170.0579, found 170.0579 $[M]^+$.

6-(Bromomethyl)-4-(methoxymethoxy)-2*H*-pyran-2-one (62)



The same method as for the preparation of **28** was employed. Thus, reaction of **61** (1.00 g, 5.88 mmol) with AIBN (96.5 mg, 588 μmol) and NBS (1.15 g, 6.46 mmol) in dry CCl_4 (50 mL) at (80°C) for 24 h afforded **62** (308.5 mg, 21%) after purification by flash chromatography (SiO_2, 1:1/EtOAc:pentane) (SiO_2, 2:1/EtOAc:pentane, R_f 0.62). IR (microscope) 2964, 1725, 1648, 1567, 1248, 1160, 1004 cm^{-1}; UV (CH_2Cl_2 solution) λ_{max} 298, 222 nm; ^1H NMR ($CDCl_3$, 600 MHz) δ 6.09 (d, 1H, $J = 2.1$ Hz, H-5), 5.55 (d, 1H, $J = 2.1$ Hz, H-3), 5.62 (s, 2H, $OC\underline{H}_2O$), 4.07 (s, 2H, H-1′), 3.41 (s, 3H, $OC\underline{H}_3$); ^{13}C NMR ($CDCl_3$, 150 MHz) δ 167.9 (C-4), 161.7 (C-2), 159.0 (C-6), 102.1 (C-5), 94.4 ($O\underline{C}H_2O$), 92.5 (C-3), 57.3 ($O\underline{C}H_3$), 26.8 (C-1′); HRMS (EI) calcd for $C_8H_9BrO_4$ 247.9684 + 249.9663, found 247.9684 + 249.9663 $[M]^+$.

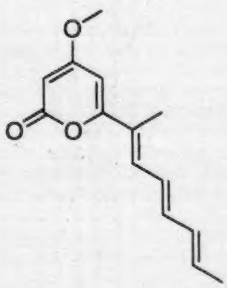
4′-Hydroxy-6′-methyl-3,4,5,6-tetrahydro-2*H*,2′*H*-2,3′-bipyran-2′-one (64)



3,4-Dihydropyran (1.19 mL, 13.4 mmol) was added dropwise to a cooled (0 °C) stirred suspension of 4-hydroxy-6-methyl-2-pyrone (**20**) (1.5 g, 11.9 mmol) in dry CH_2Cl_2 (20 mL) followed by the addition of a catalytic amount of *p*-TsOH. The reaction mixture was warmed to rt for 19 h before Et_3N and H_2O was added and the two layers separated. The aqueous layer was back extracted with CH_2Cl_2. The organic fractions were combined, dried (Na_2SO_4), filtered, and the solvent evaporated under reduced pressure. The mixture was purified by flash chromatography (SiO_2, 2:1/EtOAc:pentane, R_f 0.66) to yield **64** as an impure mixture. This was recrystallized with EtOAc to yield **64** (718.6 mg, 29%). An x-ray structure was

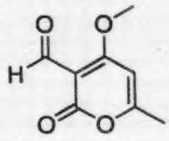
obtained to confirm structure. IR (microscope) 3350-2700 (br), 3159, 3088, 2943, 2922, 2859, 1697, 1656, 1597 cm^{-1}; ^{1}H NMR ($CDCl_3$, 600 MHz) δ 9.89 (s, 1H, O$\underline{H}$), 5.73 (s, 1H, H-5′), 4.62 (dd, 1H, *J* = 10.8, 1.8 Hz, H-2), 4.17-4.12 (m, 1H, 1 X H-6), 3.59-3.53 (m, 1H, 1 X H-6), 2.14 (s, 3H, 6′-CH_3), 2.06-2.01 (m, 1H, 1 X H-3), 1.87-1.82 (m, 1H, 1 X H-4), 1.67-1.55 (m, 3H, 1 X H-4 & H-5), 1.48-1.41 (m, 1H, 1 X H-3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 166.9 (C-4′), 163.4 (C-2′), 161.5 (C-6′), 129.4 (C-3′) 101.1 (C-5′), 77.9 (C-2), 69.6 (C-6), 30.7 (C-3), 25.8 (C-5), 22.8 (C-4), 19.7 (6′-CH_3); HRMS (EI) calcd for $C_{11}H_{14}O_4$ 210.0892, found 210.0894 $[M]^+$.

4-Methoxy-6-[(1*E*,3*E*,5*E*)-1-methylhepta-1,3,5-trien-1-yl]-2*H*-pyran-2-one (67)



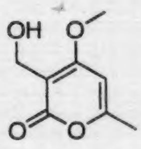
The same method as for the preparation of **27** was employed. Thus, reaction of the **3** (7.1 mg, 32.5 μmol) with K_2CO_3 (19.5 mg, 146.3 μmol) and MeI (9 μL, 146.3 μmol)) in dry acetone (2 mL) for 14 h afforded **67** (1.5 mg, 20%) after purification by flash chromatography (SiO_2, 1:1/EtOAc:pentane, R_f 0.69). IR (CH_2Cl_2 cast) 2924, 2853, 1720, 1622, 1550, 1452, 1408, 1231, 1024 cm^{-1}; UV (CH_2Cl_2 solution) λ_{max} 353, 274, 254, 220 nm; ^{1}H NMR (CD_2Cl_2, 600 MHz) δ 7.08 (d, 1H, *J* = 11.0 Hz, H-2′), 6.60-6.44 (m, 2H, H-3′ & H-4′), 6.27-6.16 (m, 1H, H-5′), 5.98 (d, 1H, *J* = 2.1 Hz, H-5), 5.92 (dq, 1H, *J* = 14.0, 6.6 Hz, H-6′), 5.41 (d, 1H, *J* = 2.1 Hz, H-3), 3.80 (s, 3H, OC$\underline{H}_3$), 1.95 (s, 3H, 1′-C$\underline{H}_3$), 1.83 (dd, 3H, *J* = 6.6, 1.2 Hz, H-7′); ^{13}C NMR (CD_2Cl_2, 150 MHz) δ 171.4 (C-4), 164.0 (C-2), 161.0 (C-6), 139.0 (C-4′), 133.8 (C-6′), 132.4 (C-2′), 132.1 (C-5′), 126.1 (C-3′), 98.5 (C-5), 88.3 (C-3 & C-1′), 56.2 (O$\underline{C}H_3$), 18.7 (C-7′), 13.0 (1′-$\underline{C}H_3$); HRMS (EI) calcd for $C_{14}H_{16}O_3$ 232.1099, found 232.1100 $[M]^+$.

4-Methoxy-6-methyl-2-oxo-2*H*-pyran-3-carbaldehyde (69)



The known compound **69**[122] was prepared using a modification of the method of Poulton and Cyr.[103] $TiCl_4$ (196 μL, 1.78 mmol) was added to a cooled (0 °C) stirred solution of **27** (50 mg, 356 μmol) in dry CH_2Cl_2 (1 mL) and the reaction mixture stirred for 15 min, at which time the mixture turned a deep orangy red. Then DCME (164 μL, 1.78 mmol) was added and the reaction mixture purged with argon to remove the HCl (g). Dry CH_2Cl_2 (2 mL) was added and the reaction mixture stirred for 14 h (colour changed to a light orange). The reaction mixture was diluted with ice and CH_2Cl_2 and the layers separated. The aqueous fraction was back extracted with CH_2Cl_2 (twice). The organic fractions were combined, washed with sat'd $NaHCO_3$, and brine, dried (Na_2SO_4), filtered and the solvent evaporated under reduced pressure. The mixture was purified by flash chromatography (SiO_2, 2:1/EtOAc:pentane, R_f 0.09) to yield **69** (20.9 mg, 35%) as a white solid. ^{1}H NMR ($CDCl_3$, 600 MHz) δ 10.10 (s, 1H, CHO), 6.13 (s, 1H, H-5), 4.02 (s, 3H, OCH$_3$), 2.32 (s, 3H, H-1′); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 186.5 (CHO), 174.3 (C-4), 170.8 (C-2), 161.9 (C-6), 101.7 (C-3), 95.0 (C-5), 57.8 (OCH$_3$), 21.5 (C-1′); HRMS (EI) calcd for $C_8H_8O_4$ 168.0423, found 168.0420 $[M]^+$.

3-(Hydroxymethyl)-4-methoxy-6-methyl-2*H*-pyran-2-one (70) (Method a)



The known compound **70**[122,149] was prepared using the method of Oikawa *et al.*[122] $NaBH_4$ (4.5 mg, 357 μmol) was added to a stirred solution of **69** (20 mg, 120 μmol) in dry MeOH (3 mL) and the reaction mixture stirred at rt for 2 h before diluting with acetone. The solvents were evaporated under reduced pressure.

The mixture was purified by flash chromatography (SiO_2, 1:4/acetone:CH_2Cl_2, R_f 0.25) to yield **70** (15.5 mg, 76%) as a white solid. If the crude aldehyde was used in this reaction, the acetal **71** was obtained in place of the product. IR (microscope) 3421 (br), 3099, 2953, 1702, 1645, 1563, 1469, 13990, 1353, 1264 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 6.03 (s, 1H, H-5), 4.50 (s, 2H, $C\underline{H}_2OH$), 3.87 (s, 3H, $OC\underline{H}_3$), 2.90 (bs s, 1H, $CH_2O\underline{H}$), 2.26 (s, 3H, H-1'); ^{13}C NMR ($CDCl_3$, 150 MHz) δ 167.1 (C-4), 165.5 (C-2). 163.3 (C-6), 103.7 (C-3), 94.5 (C-5), 56.0 ($\underline{C}H_3$), 54.2 ($\underline{C}H_2OH$), 20.3 (C-1'); HRMS (EI) calcd for $C_8H_{10}O_4$ 170.0579, found 170.0575 $[M]^+$.

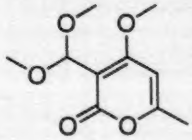
3-(Hydroxymethyl)-4-methoxy-6-methyl-2*H*-pyran-2-one (70) (Method b)

$TiCl_4$ (196 μL, 1.78 mmol) was added to a stirred solution of **27** (50 mg, 356 μmol) in dry CH_2Cl_2 (2 mL) and the reaction mixture stirred at (0 °C) for 5 min before bubbling H_2CO (obtained by heating *para*-formaldehyde) through the reaction mixture for 45 min and allowing to warm to rt. H_2O was added to the reaction mixture and it was acidified with 1 N HCl (pH 2) and the mixture extracted with CH_2Cl_2 (twice). The organic fractions were combined, dried (Na_2SO_4), filtered and the solvent evaporated under reduced pressure. The mixture was purified by flash chromatography (SiO_2, 2:1/EtOAc:pentane) switching to 9:1/EtOAc:pentane and finally to 5% MeOH in EtOAc to elute the product **70** (13.1 mg, 22 %) as a white solid.

3-(Hydroxymethyl)-4-methoxy-6-methyl-2*H*-pyran-2-one (70) (Method c)

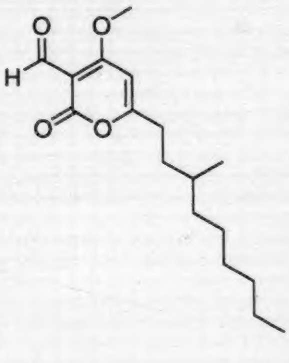
The same method and amounts as for the preparation of **70** (Method b) was employed with the exception that *para*-formaldehyde (50 mg) was added instead of bubbling formaldehyde through the reaction mixture. The reaction mixture was allowed to stir for 1.5 h before it was quenched as described for **70** (Method b) and purified as for **70** (Method a) to yield **70** (16 mg, 26%) as white crystals. An x-ray structure was obtained.

3-(Dimethoxymethyl)-4-methoxy-6-methyl-2*H*-pyran-2-one (71)



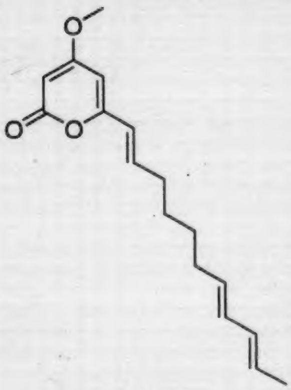
The same method as for the preparation of **70** (Method a) was employed, except using unpurified aldehyde **69** to yield **71** (22.4 mg) (SiO_2, 2:1/EtOAc:pentane, R_f 0.10). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 6.00 (s, 1H, H-5), 5.41 (s, 1H, CH(OCH_3)$_2$), 3.89 (s, 3H, OCH$_3$), 3.41 (s, 6H, CH(OCH$_3$)$_2$), 2.23 (s, 3H, C-1′); HRMS (EI) calcd for $C_{10}H_{14}O_5$ 214.0841, found 214.0833 $[M]^+$.

4-Methoxy-6-(3-methylnonyl)-2-oxo-2*H*-pyran-3-carbaldehyde (72)



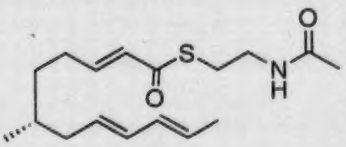
$TiCl_4$ (21 μL, 187 μmol) was added to a cooled (0°C) stirred solution of **31** (50 mg, 187 μmol) in DCME (500 μL, 5.5 mmol) and the reaction mixture stirred for 3 h (HCl gas produced turned the pH paper red). The reaction mixture went dry, so another 500 μL of DCME was added and the reaction mixture left to stir overnight while warming to rt. The reaction mixture went dry overnight, thus Et_2O, ice and H_2O were added. The layers were separated and the aqueous fractions back extracted with Et_2O. The ethereal layers were combined, washed with brine, dried (Na_2SO_4), filtered and the solvent evaporated under reduced pressure. By ^{1}H-NMR, less than 1% product was observed with the majority of the material being clean starting pyrone. The reaction was restarted with the addition of DCME (750 μL) and $TiCl_4$ (100 μL) in CH_2Cl_2 (2 mL) for 3 days before working up as above and purifying by flash chromatography (SiO_2, 1:1 EtOAc:pentane) to yield **72** (3.7 mg, 7%) (SiO_2, 2:1 EtOAc:pentane, R_f 0.39). IR (CH_2Cl_2 cast) 3102, 3025, 2956, 2855, 2771, 1753, 1689, 1655 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 10.11 (d, 1H, *J* = 0.8 Hz, C$\underline{H}$O), 6.08 (s, 1H, H-5), 4.04 (s, 3H, OC$\underline{H}_3$), 2.64-2.45 (m, 2H, H-1′), 1.75-1.65 (m, 1H, 1 X H-2′), 1.54-1.40 (m, 2H, 1 X H-2′ & H-3′), 1.33-1.21 (m, 9H, 1 X H-4′ & H-5′ & H-6′ & H-7′ & H-8′), 1.17-1.09 (m, 1H, 1 X H-4′), 0.90 (d, 3H, *J* = 6.4 Hz, 3′-$\underline{C}H_3$), 0.86 (t, 3H, *J* = 6.8 Hz, C-9′); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 186.7 ($\underline{C}$HO) 174.8 (C-4), 170.0 (C-2), 162.4 (C-6), 101.9 (C-3), 93.9 (C-5), 57.6 (O$\underline{C}H_3$), 36.6 (C-1′), 33.8 (C-6′), 33.1 ($\underline{C}H_2$), 32.5 (C-3′), 31.8 ($\underline{C}H_2$), 29.5 (C-7′), 26.8 (C-2′), 22.6 (C-8′), 19.3 (3′-$\underline{C}H_3$), 14.1 (C-9′); HRMS (EI) calcd for $C_{17}H_{26}O_4$ 294.1831, found 294.1828 $[M]^+$.

4-Methoxy-6-[(1*E*,7*E*,9*E*)-undeca-1,7,9-trien-1-yl]-2*H*-pyran-2-one (73)



The same method as for the preparation of **31** was employed. Thus, reaction of **29** (343.2 mg, 713 μmol) with *n*-BuLi (1.6 M in hexanes, 490 μL, 784 μmol) for 6 h, followed by addition of the aldehyde (Scheme 20) (86.1 mg, 648 μmol) in dry THF (10 mL) at rt overnight afforded **73** (3.3 mg, 21%) after purification by flash chromatography, 3 columns (SiO_2, 2:1/EtOAc:pentane), then (SiO_2, 1:2/EtOAc:pentane) then (SiO_2, 1:4/EtOAc:pentane), (SiO_2, 1:1/EtOAc:pentane, R_f 0.53). IR (microscope) 3405 (br), 3091, 2933, 2858, 1721, 1655, 1617, 1557, 1455, 1409, 1253 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 6.67 (dt, 1H, *J* = 15.5, 7.0 Hz, H-2′), 6.03-5.94 (m, 2H, H-8′ & H-9′), 5.91 (dt, 1H, *J* = 15.5 1.4 Hz, H-1′), 5.73 (d, 1H, *J* = 2.0 Hz, H-5), 5.56 (dq, 1H, *J* = 13.5, 6.5 Hz, H-10′), 5.51 (dt, 1H, *J* = 14.0, 7.0 Hz, H-7′), 5.41 (d, 1H, *J* = 2.0 Hz, H-3), 3.78 (s, 3H, OCH$_3$), 2.18 (ddt, 2H, *J* = 7.0, 7.0, 1.4 Hz, H-3′), 2.04 (dt, 2H, *J* = 7.0, 7.0 Hz, H-6′), 1.71 (d, 3H, *J* = 6.5, H-11′), 1.49-1.35 (m, 4H, H-4′ & H-5′); ^{13}C NMR (CDCl$_3$, 125 MHz) δ 171.2 (C-4), 159.7 (C-2), 150.8 (C-6), 140.0 (C-2′), 131.5 (CH), 130.6 (CH), 127.0 (CH), 121.4 (C-1′), 114.9 (CH), 99.5 (C-5), 88.3 (C-3), 55.8 (OCH$_3$), 32.6 (C-3′), 32.3 (C-6′), 28.9 (CH$_2$), 28.0 (CH$_2$), 18.1 (C-11′); HRMS (EI) calcd for $C_{17}H_{22}O_3$ 274.1569, found 274.1566 [M]$^+$.

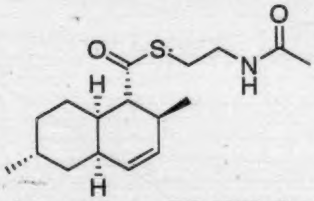
S-[2-(Acetylamino)ethyl] (2*E*,6*R*,8*E*,10*E*)-6-methyldodeca-2,8,10-trienethioate (74)



The known compound **74**[73,124] was prepared using the method described by Van den Heever.[124] LiBr (52 mg, 0.60 mmol) and distilled Et$_3$N (83 μL, 0.6 mmol) were added to a cooled (0 °C) solution of **97** (196 mg, 0.66 mmol) in dry CH$_2$Cl$_2$ (9 mL). The reaction mixture was allowed to warm to rt before the aldehyde **109** (100 mg, 0.6 mmol) in dry CH$_2$Cl$_2$ (1 mL) was added. The reaction mixture was stirred at rt overnight, before the solvent was removed under reduced pressure. The resulting mixture was purified by flash chromatography (SiO$_2$, 5% MeCN in EtOAc, R_f 0.45) to yield **74** (65 mg, 35%) as a white solid. $[\alpha]^{20}_{D}$ -3.65° (*c* 0.40, CHCl$_3$) (lit.[73] $[\alpha]^{20}_{D}$ -7.41° (*c* 0.08, CH$_2$Cl$_2$)); IR (microscope) 3313, 3085, 3010, 2960, 2906, 2849, 2833, 1667, 1632, 1548, 1449, 1291, 1201, 1074 cm^{-1}; ^{1}H NMR (CDCl$_3$, 300 MHz) δ 6.90 (dt, 1H, *J* = 15.6, 6.9 Hz, H-3), 6.10 (dt, 1H, *J* = 15.6, 1.5 Hz, H-2), 6.06-5.93 (m, 2H, H-9 & H-10), 5.90-5.80 (br s, 1H, NH), 5.57 (dq, 1H, *J* = 13.4, 6.6 Hz, H-11), 5.47 (dt, 1H, *J* = 14.4, 7.2 Hz, H-8), 3.44 (dt, 2H, *J*

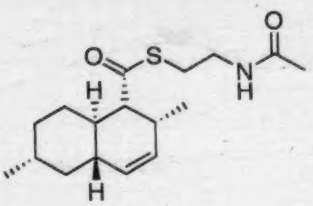
= 6.0, 6.0 Hz, H-3′), 3.06 (t, 2H, *J* = 6.0 Hz, H-2′), 2.23-2.13 (m, 2H, H-4), 2.04 (ddd, 1H, *J* = 14.0, 7.2, 6.9 Hz, 1 X H-7), 1.90 (ddd, 1H, *J* = 14.0, 7.2, 7.0 Hz, 1 X H-7), 1.94 (s, 3H, H-6′), 1.71 (d, 3H, *J* = 6.6 Hz, H-12), 1.58-1.38 (m, 2H, 1 X H-5, & H-6), 1.34-1.18 (m, 1H, 1 X H-5), 0.86 (d, 3H, *J* = 6.6 Hz, 6-C$\underline{H}_3$); ^{13}C NMR (CDCl$_3$, 125 MHz) δ 190.3 (C-1), 170.3 (C-5′), 146.7 (C-3), 131.9 (C-9), 131.5 (C-10), 129.6 (C-8), 128.2 (C-2), 127.2 (C-11), 39.8 (C-7), 39.8 (C-3′), 34.5 (C-4), 32.8 (C-6), 29.9 (C-5), 28.3 (C-2′), 23.2 (C-6′), 19.3 (6-$\underline{C}H_3$), 18.0 (C-12); HRMS (EI) calcd for $C_{17}H_{27}NO_2S$ 309.1762, found 309.1761 [M]$^+$.

S-[2-(Acetylamino)ethyl] (1*R*,2*S*,4a*R*,6*R*,8a*R*)-2,6-dimethyl-1,2,4a,5,6,7,8,8a-octahydronaphthalene-1-carbothioate (*exo*) (75)



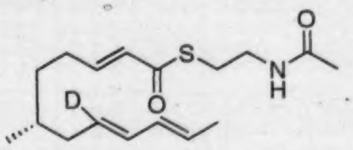
75 was prepared using the same reaction as for the synthesis of **76**, **75** was also isolated by HPLC using the same method and column as for **76**. The title compound **75** (1.4 mg, 5%) eluted from RP-HPLC at (t_r 23.5 min). ^{1}H-NMR corresponded to literature values.[73] ^{1}H NMR (CDCl$_3$, 500 MHz) δ 5.78 (br s, 1H, N$\underline{H}$), 5.53 (ddd, 1H, *J* = 10.0, 4.0, 2.5 Hz, H-3), 5.41 (ddd, 1H, *J* = 10.0, 2.0, 2.0 Hz, H-4), 3.48-3.40 (m, 2H, H-4′), 3.01 (t, 2H, *J* = 6.5 Hz, H-3′), 2.60 (dd, 1H, *J* = 9.5, 8.5 Hz, H-1), 2.56-2.48 (m, 1H, H-2), 2.32-2.25 (m, 1H, H-4a), 2.15-2.04 (m, 1H, H-8a), 1.94 (s, 3H, H-7′), 1.90-1.80 (m, 1H, H-6), 1.73-1.57 (m, 2H, H-7eq, H-8eq), 1.43-1.32 (m, 3H, H-5eq & H-5ax & H-8ax), 1.18-1.12 (m, 1H, H-7ax), 1.00 (d, 3H, *J* = 7.0 Hz, 2-C$\underline{H}_3$), 0.95 (d, 3H, *J* = 7.5 Hz, 6-C$\underline{H}_3$).

S-[2-(Acetylamino)ethyl] (1*R*,2*R*,4a*S*,6*R*,8a*R*)-2,6-dimethyl-1,2,4a,5,6,7,8,8a-octahydronaphthalene-1-carbothioate (*endo*) (76)



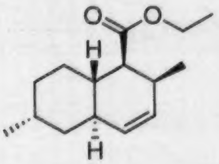
The known compound **76**[73,77] was prepared using a modified version of the method described by Witter.[73,150] **74** (29.2 mg, 94.3 μmol) Was dissolved in 250 μL EtOH and pellet buffer (pH 7.8) (the buffer is described in the fermentation section below) and left to stir for 2.5 days at rt before heating to 50 °C for an additional 2 days. The reaction mixture was diluted with a H_2O and neutralized with 0.5 M HCl. The mixture was then extracted with $CHCl_3$ (4 X 6 mL), dried ($MgSO_4$), filtered and the solvent evaporated under reduced pressure. The mixture was then dissolved in MeCN and purified by RP-HPLC. HPLC (Waters phenyl column, MeCN and H_2O (no TFA), 1 mL/min, 206 nm), 40% MeCN was increased to 55% over 30 min followed by an increase to 80% in 0.1 min and remaining there for 4 min before lowering to 35% in 0.1 min and remaining there for an additional 4 min. The title compound **76** eluted at t_r 26 min (3.4 mg, 11%); ^{1}H-NMR corresponded to literature values.[73] ^{1}H NMR ($CDCl_3$, 500 MHz) δ 5.82 (br s, 1H, NH), 5.51 (ddd, 1H, *J* = 10.0, 5.0, 3.0 Hz, H-3), 5.37 (d, 1H, *J* = 10.0 Hz, H-4), 3.46-3.37 (m, 2H, H-4′), 3.01 (t, 2H, *J* = 6.5 Hz, H-3′), 2.84 (dd, 1H, *J* = 11.5, 6.0 Hz, H-1), 2.60-2.53 (m, 1H, H-2), 1.95 (s, 3H, H-7′), 1.82-1.66 (m, 4H, H-4a & H-5eq & H-7eq & H-8eq), 1.50-1.40 (m, 2H, H-6 & H-8a), 1.04-0.90 (m, 2H, H-7ax & H-8ax), 0.88 (d, 3H, *J* = 6.5 Hz, 2-CH_3), 0.87 (d, 3H, *J* = 7.0 Hz, 6-CH_3), 0.75 (ddd, 1H, *J* = 12.5, 12.5, 12.5 Hz, H-5ax).

S-[2-(Acetylamino)ethyl] (2E,6R,8E,10E)-6-methyldodeca-2,8,10-trienethioate-d_1 (78)



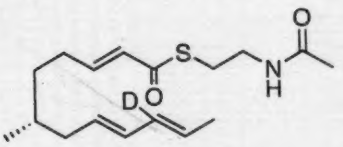
The same method as for the preparation of unlabeled **74** was employed. Thus, reaction of the labeled **95** (75 mg, 447 μmol) with **97** (133 mg, 447 μmol), LiBr (39 mg, 447 μmol), Et_3N (63 μL, 447 μmol) in CH_2Cl_2 afforded **78** (59.1 mg, 42%) as a white solid after purification. This material was stored dry, under argon at -80 °C and showed no cyclisation by ^{1}H-NMR after 2 yrs. $[\alpha]_D^{20}$ -5.18° (*c* 0.44, $CHCl_3$); IR (microscope) 3133, 3085, 3007, 2961, 2907, 2871, 2849, 2219, 1668, 1649, 1549, 1448, 1291, 1074 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 6.90 (dt, 1H, *J* = 15.3, 6.9 Hz, H-3), 6.10 (dt, 1H, *J* = 15.3, 1.5 Hz, H-2), 6.06-5.90 (m, 2H, H-9 & H-10), 5.90-5.82 (br s, 1H, NH), 5.62-5.50 (m, 1H, H-11), 3.44 (dt, 2H, *J* = 6.0, 6.0 Hz, H-3′), 3.06 (t, 2H, *J* = 6.0 Hz, H-2′), 2.31-2.08 (m, 2H, H-4), 2.04 (dd, 1H, *J* = 15.0, 6.3 Hz, 1 X H-7), 1.90 (dd, 1H, *J* = 14.1, 7.0 Hz, 1 X H-7), 1.94 (s, 3H, H-6′), 1.71 (d, 3H, *J* = 6.6 Hz, H-12), 1.58-1.38 (m, 2H, 1 X H-5, & H-6), 1.34-1.18 (m, 1H, 1 X H-5), 0.86 (d, 3H, *J* = 6.6 Hz, 6-C$\underline{H}_3$); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 190.2 (C-1), 170.3 (C-5′), 146.6 (C-3), 131.8 (C-9), 131.4 (C-10), 129.2 (t, *J* = 23.3 Hz, C-8), 128.1 (C-2), 127.0 (C-11), 39.6 (C-7), 39.6 (C-3′), 34.4 (C-4), 32.7 (C-6), 29.8 (C-5), 28.1 (C-2′), 23.0 (C-6′), 19.2 (6-$\underline{C}H_3$), 17.9 (C-12); HRMS (EI) calcd for $C_{17}H_{26}DNO_2S$ 310.1824, found 310.1825 $[M]^+$.

Ethyl (1*S*,2*S*,4a*R*,6*R*,8a*S*)-2,6-dimethyl-1,2,4a,5,6,7,8,8a-octahydronaphthalene-1-carboxylate (79)



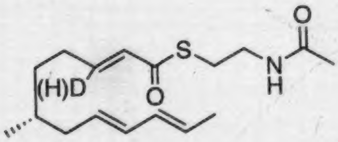
The known compound **79**[73] was prepared using a modified version of the method of Gutierrez *et al.*[151] **127** (279.8 mg, 857 μmol) Had argon bubbled through it for 30 min. AIBN (10 mg) was added followed by 10 min of argon bubbling and the addition of Bu_3SnH (692 μL, 2.57 mmol). The reaction mixture was heated to 120 °C with a condenser attached for 24 h. The mixture was cooled and filtered though a plug (SiO_2, 1:1/Et_2O:pentane). The crude material was then purified by flash chromatography (SiO_2, 2% Et_2O in pentane) to yield pure **79** (183.5 mg, 90%) (SiO_2 5% Et_2O in pentane, R_f 0.51). GC-MS (t_r 14.52 min); $[\alpha]_D^{20}$ +99.29° (*c* 0.35, $CHCl_3$) (lit.[73] $[\alpha]_D^{20}$ +143.3° (*c* 0.72, $CHCl_3$)); IR (microscope) 3012, 2960, 2911, 2850, 1736, 1654, 1459 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 5.53 (ddd, 1H, *J* = 9.8, 4.4, 3.2 Hz, H-3), 5.30 (d, 1H, *J* = 9.8 Hz, H-4), 4.12 (q, 2H, *J* = 7.2 Hz, $OC\underline{H}_2CH_3$), 2.58-2.50 (m, 2H, H-1 & H-2), 2.08-1.98 (m, 1H, H-6), 1.96-1.86 (m, 1H, H-4a), 1.70 (dddd, 1H, *J* = 12.4, 3.6, 3.6, 3.2 Hz, H-8eq), 1.62 (dddd, 1H, *J* = 13.6, 13.2, 4.4, 4.4 Hz, H-7ax), 1.52-1.48 (m, 1H, H-7eq), 1.48-1.44 (m, 1H, H-5eq), 1.40-1.33 (m, 1H, H-8a), 1.32 (ddd, 1H, *J* = 13.2, 13.2, 4.8 Hz, H-5ax), 1.24 (t, 3H, *J* = 7.2 Hz, $OCH_2C\underline{H}_3$), 1.08 (dddd, 1H, *J* = 12.4, 12.4, 12.0, 3.6 Hz, H-8ax), 0.97 (d, 3H, *J* = 7.2 Hz, 6-$C\underline{H}_3$), 0.90 (d, 3H, *J* = 7.2 Hz, 2-$C\underline{H}_3$); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 173.8 ($\underline{C}$=O), 131.1 ($\underline{C}$H=CH), 131.1 ($\underline{C}$H=CH), 59.7 ($O\underline{C}H_2CH_3$), 49.6 (C-1), 38.7 (C-5), 37.1 (C-8a), 35.6 (C-4a), 32.3 (C-2), 31.9 (C-7), 27.6 (C-6), 24.4 (C-8), 18.3 (6-$\underline{C}H_3$), 17.7 (2-$\underline{C}H_3$), 14.3 ($OCH_2\underline{C}H_3$); HRMS (EI) calcd for $C_{15}H_{24}O_2$ 236.1776, found 236.1778 $[M]^+$.

S-[2-(Acetylamino)ethyl] (2*E*,6*R*,8*E*,10*E*)-6-methyldodeca-2,8,10-trienethioate-d_1 (81)



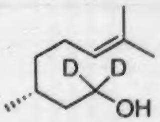
81 Was prepared by Dr. Belén Mayo-Martín[128] using the same method as for the preparation of **74**. LiBr (41 mg, 0.48 mmol) was added to a solution of **97** (142 mg, 0.48 mmol) in freshly distilled CH_2Cl_2 (5 mL), which turned the solution colour to orange. The mixture was stirred at rt for 25 min before the mixture was cooled to 0 °C and Et_3N (66 μL, 0.48 mmol) was added. The aldehyde **107** (40 mg, 0.24 mmol) in freshly distilled CH_2Cl_2 (5 mL) was added to the reaction mixture *via* a cannula. The reaction mixture was stirred and allowing to warm to rt over 1.5 h. The solvents were evaporated under reduced pressure to give an orange liquid which was purified by flash chromatography (SiO_2, EtOAc, R_f 0.25) to yield **81** (7 mg, 10%) as a colourless oil. $[\alpha]_D^{20}$ −10.54° (*c* 0.11, CH_2Cl_2); IR ($CHCl_3$ cast) 1743 (s), 1674 (m) cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 6.96-6.87 (m, 1H, H-3), 6.14-6.08 (m, 1H, H-2), 5.97 (d, 1H, *J* = 15.4 Hz, H-9), 5.62-5.52 (m, 1H, H-11), 5.51-5.44 (m, 1H, H-8), 3.46 (apparent q, 2H, *J* = 6.8 Hz, H-3′), 3.05 (t, 2H *J* = 6.8 Hz, H-2′), 2.30-1.85 (m, 4H, H-7 & H-4), 1.92 (s, 3H, H-6′), 1.74 (d, 3H, *J* = 6.7 Hz, H-12), 1.70-1.43 (m, 2H, 1 X H-5 & H-6), 1.30-1.15 (m, 1H, 1 X H-5), 0.87 (d, 3H, *J* = 6.5 Hz, 6-C$\underline{H}_3$); HRMS (ES) calcd $C_{17}H_{26}DNO_2S$ 310.1824, found 333.1714 $[M+Na]^+$.

S-[2-(Acetylamino)ethyl] (2*E*,6*R*,8*E*,10*E*)-6-methyldodeca-2,8,10-trienethioate-d_1 (83)



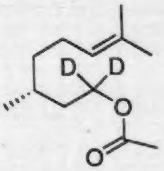
83 Was prepared by Dr. Belén Mayo-Martín[128] using the same method used for the preparation of **74**. LiBr (30 mg, 0.36 mmol) was added to a solution of **97** (105 mg, 0.36 mmol) in freshly distilled CH_2Cl_2 (8 mL), which turned the solution colour to orange. The mixture was stirred at rt for 55 min before cooling the mixture to 0 °C and adding Et_3N (50 μL, 0.36 mmol) and letting stir for 25 min. The aldehyde **111** (20 mg, 0.12 mmol) in freshly distilled CH_2Cl_2 (3 mL) was added to the reaction mixture *via* a cannula. The reaction mixture was stirred for 1 h before the solvents were evaporated under reduced pressure to give an orange liquid which was purified by flash chromatography (SiO_2, EtOAc, R_f 0.25) to yield **83** (10 mg, 27%) as a colourless oil. The ratio of non-deuterated:deuterated aldehyde was 40: 60. $[\alpha]_D^{20}$ –6.55 (*c* 0.18, CH_2Cl_2); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 6.12 (s, 1H, H-2), 6.07-5.96 (m, 2H, H-9 & H-10), 5.85 (br s, 1H, H-4′), 5.66-5.55 (m, 1H, H-11), 5.54-5.45 (m, 2H, H-8), 3.47 (apparent q, 2H, *J* = 5.9 Hz, H-3′), 3.10 (t, 2H, *J* = 6.2 Hz, H-2′), 2.30-2.13 (m, 2H, H-4), 2.13-2.03 (m, 1H, 1 X H-7), 1.97 (s, 1H, H-6′), 1.98-1.90 (m, 1H, 1 X H-7), 1.74 (d, 3H, *J* = 7.2 Hz, H-12), 1.65-1.48 (m, 2H, 1 X H-5 & H-6), 1.35-1.25 (m, 1H, 1 X H-5), 0.90 (d, 3H, *J* = 6.6 Hz, 6-C$\underline{H}_3$); HRMS (ES) calcd $C_{17}H_{26}DNO_2S$ 310.1824, found 333.1714 $[M+Na]^+$.

(3*R*)-3,7-Dimethyloct-6-en-1-ol-*d*$_2$ (86)



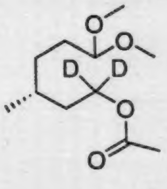
The known compound **86**[125] was prepared using a modified version of the method described by White *et al.*[152] Neat *R*-(+)-citronellic acid (9.8036 g, 57.60 mmol) was added to a cooled (0 °C) stirred solution of $LiAlD_4$ (57.60 mL of a 1 M solution in Et_2O, 57.60 mmol) dropwise over 45 min and the reaction mixture was allowed to stir and warm to rt overnight. NaOH (1 M, 20mL) was added to the reaction mixture and it was left stirring for 30 min before filtering the aluminum salts through a bed of celite and the celite washed with EtOAc. The organics were washed with sat'd $NaHCO_3$ and brine. The aqueous fractions were combined and back extracted with EtOAc. The organic layers were combined, dried (Na_2SO_4), filtered and the solvent evaporated under reduced pressure to yield **86** in quantitative yield. (R_f 0.41 on SiO_2, 1:1/Et_2O:pentane). $[\alpha]_D^{20}$ + 4.66 (*c* 1.63, $CHCl_3$); IR ($CHCl_3$ cast) 3329 (br), 2963, 2855, 2199, 2092, 1452, 1377, 1177, 1131, 1076 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 5.07 (m, 1H, H-6), 2.05-1.86 (m, 2H, H-5), 1.65 (s, 3H, HC=C$\underline{H}_3$), 1.57 (s, 3H, HC=C$\underline{H}_3$), 1.62-1.47 (m, 2H, 1 X H-2, & H-3), 1.40-1.22 (m, 2H, 1 X H-2, & 1 X H-4), 1.20-1.08 (m, 1H, 1 X H-4), 0.87 (d, 3H, J = 6.6, 3-C$\underline{H}_3$); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 131.3 (C-7), 124.7 (C-6), 60.6 (m, C-1), 39.7 (C-2), 37.3 (C-4), 29.2 (C-3), 25.7 (7-$\underline{C}H_3$), 25.5 (C-5), 19.5 (3-$\underline{C}H_3$), 17.6 (7-$\underline{C}H_3$); HRMS (EI) calcd for $C_{10}H_{18}D_2O$ 158.1638, found 158.1646 $[M]^+$.

(3R)-3,7-Dimethyloct-6-en-1-yl acetate-d$_2$ (87)



The same method as for the preparation of unlabeled **99** was employed. Thus, reaction of the labeled alcohol **86** (2.4296 g, 15.35 mmol) with acetyl chloride (4.38 mL, 61.40 mmol) and diisopropylamine (4.303 mL, 30.70 mmol) afforded **87** (3.000 g, 98%) after purification by flash chromatography. $[\alpha]^{20}_{\mathrm{D}}$ −3.64 (*c* 1.95, $CHCl_3$); IR ($CHCl_3$ cast) 2963, 2925, 2871, 2240, 2160, 1741, 1454, 1369, 1258, 1161, 1058, 1030 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 5.10-5.00 (m, 1H, H-6), 2.02 (s, 3H, H-2′), 2.04-1.90 (m, 2H, H-5), 1.70-1.58 (m, 1H, 1 X H-2), 1.66 (s, 3H, $\mathrm{HC{=}C\underline{H}_3}$), 1.58 (s, 3H, $\mathrm{HC{=}C\underline{H}_3}$), 1.58-1.49 (m, 1H, H-3), 1.47-1.39 (m, 1H, 1 X H-2), 1.38-1.26 (m, 1H, 1 X H-4), 1.22-1.10 (m, 1H, 1 X H-4), 0.89 (d, 3H, *J* = 6.6 Hz, 3-$\mathrm{C\underline{H}_3}$); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 171.3 (C-1′), 131.4 (C-7), 124.6 (C-6), 61.5 (C-1), 37.0 (C-2), 35.3 (C-4), 29.5 (C-3), 25.7 (7-$\mathrm{\underline{C}H_3}$), 25.4 (C-5), 21.1 (C-2′), 19.5 (3-$\mathrm{\underline{C}H_3}$), 17.7 (7-$\mathrm{\underline{C}H_3}$); HRMS (EI) calcd for $C_{12}H_{20}D_2O_2$ 200.1743, found 200.1741 $[M]^+$.

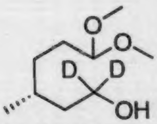
(3*R*)-6,6-Dimethoxy-3-methylhexyl acetate-*d*$_2$ (88)



The same method as for the preparation of unlabeled **100** was employed. Thus, reaction of the labeled acetate **87** (3.000 g, 15.13 mmol) with O_3 (35 min), Ar (35 min), *p*-TsOH (287 mg, 1.51 mmol) and dimethyl sulfide (2.22 mL, 30.26 mmol) afforded **88** (2.5147 g, 75%) after purification by flash chromatography. $[\alpha]^{20}_{\mathrm{D}}$ +2.10° (*c* 1.21, $CHCl_3$); IR (microscope) 2954, 2933, 2830, 2237, 2157, 1739, 1460, 1371, 1262, 1132 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 4.31 (t, 1H, *J* = 5.7 Hz, H-6), 3.29 (s, 6H, 2 X $\mathrm{OC\underline{H}_3}$), 2.01 (s, 3H, H-2′), 1.67-1.50 (m, 4H, 1 X H-2, & H-3, & H-5), 1.49-1.32 (m, 2H, 1 X H-2, & 1 X H-4), 1.24-1.10 (m, 1H, 1 X H-4), 0.89

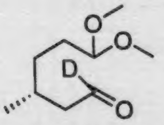
(d, 3H, *J* = 6.3 Hz, 3-CH_3); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 171.1 (C-1′), 104.7 (C-6), 62.5 (C-1), 52.7 (1 X OCH_3), 52.6 (1 X OCH_3), 35.2 ($\underline{C}H_2$), 31.5 ($\underline{C}H_2$), 29.9 ($\underline{C}H_2$), 29.7 (C-3), 20.9 (C-2′), 19.3 (3-$\underline{C}H_3$); HRMS (ES) calcd for $C_{11}H_{20}D_2O_4$ 220.1642, found 243.1536 $[M+Na]^+$.

(3*R*)-6,6-Dimethoxy-3-methylhexan-1-ol-*d*$_2$ (89)



The same method as for the preparation of unlabeled **101** was employed. Thus, reaction of the labeled **88** (6.601 g, 29.98 mmol) with NaOMe in H_2O afforded **89** (4.9008 g, 92%). IR (microscope) 3430, 2952, 2928, 2872, 2832, 2191, 2089, 1459, 1380, 1132, 1045 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 4.31 (t, 1H, *J* = 5.7 Hz, H-6), 3.29 (s, 6H, 2 XOC$\underline{H}_3$), 1.70-1.49 (m, 4H, 1 X H-2, & H-3, & H-5), 1.45-1.29 (m, 2H, 1 X H-2, & O$\underline{H}$), 1.25-1.12 (m, 2H, H-4), 0.88 (d, 3H, *J* = 6.6 Hz, 3-C$\underline{H}_3$); ^{13}C NMR ($CDCl_3$, 150 MHz) δ 104.9 (C-6), 60.5 (C-1), 52.7 (O$\underline{C}H_3$), 52.5 (O$\underline{C}H_3$), 39.75 ($\underline{C}H_2$), 31.5 ($\underline{C}H_2$), 29.6 ($\underline{C}H_2$), 29.2 (C-3), 19.3 (3-$\underline{C}H_3$); HRMS (EI) calcd for $C_9H_{17}D_2O_3$ 177.1458, found 177.1459 $[M-H]^+$, calcd for $C_9H_{18}D_2O_3$ 178.1536, found 147.1353 $[M-OCH_3]^+$.

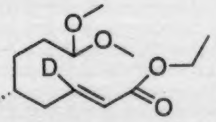
(3*R*)-6,6-Dimethoxy-3-methylhexanal-*d*$_1$ (90)



The same method as for the preparation of unlabeled **102** was employed. Thus, reaction of the labeled alcohol **89** (1.6441 g, 9.22 mmol) with DMP (4.6940 g, 11.1 mmol) afforded **90** (922.5 mg, 57%) after purification by flash chromatography. ^1H NMR ($CDCl_3$, 300 MHz) δ 4.30 (t, 1H, *J* = 5.7 Hz, H-6), 3.29 (s, 6H, 2 X OC$\underline{H}_3$), 2.38 (dd, 1H, *J* = 16.2, 5.7 Hz, 1 X H-2), 2.21 (dd, 1H, *J* = 16.2, 7.5 Hz, 1 X H-2), 2.10-1.90 (m, 1H, H-3), 1.70-1.48 (m, 2H, H-5), 1.44-1.10 (m, 2H, H-4), 0.95

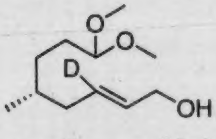
(d, 3H, J = 6.6 Hz, 3-CH$_3$); ^{13}C NMR (CDCl$_3$, 125 MHz) δ; 202.2 (m, C-1), 104.6 (C-6), 52.8 (2 X OCH$_3$), 50.8 (C-2), 31.6 (CH$_2$), 30.0 (CH$_2$), 28.0 (C-3), 19.9 (3-CH$_3$); HRMS (EI) calcd for $C_9H_{17}DO_3$ 175.1318, found 174.1238 [M-H]$^+$, 144.1133 [M-OCH$_3$]$^+$.

Ethyl (2*E*,5*R*)-8,8-dimethoxy-5-methyloct-2-enoate-*d*$_1$ (91)



The same method as for the preparation of unlabeled **103** was employed. Thus, reaction of the labeled **90** (1.7706 g, 10.1 mmol) with (carbethoxymethylene)triphenylphosphorane (4.7701 g, 11.11 mmol) afforded **91** (2.3003 g, 93%) which was used without further purification. $[\alpha]_D^{20}$ +2.32° (c 2.00, CHCl$_3$); IR (microscope) 2954, 2932, 2830, 2243, 2044, 1720, 1639, 1461, 1367, 1311, 1266, 1189, cm^{-1}; ^{1}H NMR (CDCl$_3$, 300 MHz) δ 5.80-5.76 (m, 1H, H-2), 4.30 (t, 1H, J = 5.7 Hz, H-8), 4.15 (q, 2H, J = 7.1 Hz, H-2′), 3.28 (s, 6H, 2 X OCH$_3$), 2.18 (ddd, 1H, J = 14.4, 5.4, 0.6 Hz, 1 X H-4),), 2.01 (ddd, 1H, J = 14.4, 7.5, 0.6 Hz, 1 X H-4), 1.68-1.46 (m, 3H, H-5, & H-7), 1.43-1.10 (m, 2H, H-6), 1.26 (t, 3H, J = 7.1 Hz, H-3′), 0.88 (d, 3H, J = 6.6 Hz, 5-CH$_3$); ^{13}C NMR (CDCl$_3$, 100 MHz) δ 166.6 (C-1), 147.4 (t, J = 23.8 Hz, C-3), 122.6 (C-2), 104.8 (C-8), 60.2 (C-1′), 52.8 (O-CH$_3$), 52.7 (O-CH$_3$), 39.5 (C-4), 32.5 (C-5), 31.4 (CH$_2$), 30.2 (CH$_2$), 19.5 (5-CH$_3$), 14.3 (C-2′); HRMS (EI) calcd for $C_{13}H_{23}DO_4$ 245.1736, found 244.1658 [M-H]$^+$, 214.1551 [M-OCH$_3$]$^+$.

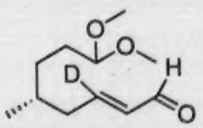
(2*E*,5*R*)-8,8-Dimethoxy-5-methyloct-2-en-1-ol-*d*$_1$ (92)



Compound **92** was prepared using a modified version of the method described by Nicolaou *et al.*[153] Neat DIBAL-H (3.51 mL, 19.70 mmol) was added to a cooled (-78 °C) solution of **91** (2.3003 g, 9.38 mmol) in dry THF

(30 mL) dropwise over 30 min and the reaction mixture was allowed to stir and warm to rt overnight. Sat'd $NaHCO_3$ (10 mL) was added to the reaction mixture and the aluminum salts removed by filtration through a pad of celite. The celite was washed with EtOAc, and the solvents were evaporated under reduced pressure and the crude mixture was purified by flash chromatography (SiO_2, 2:1/Et_2O:pentane, R_f 0.20) to yield **92** (1.7494 g, 92%). $[\alpha]_D^{20}$ +3.09° (*c* 1.95, $CHCl_3$); IR (microscope) 3429 (br), 2952, 2928, 2872, 2832, 2222, 1724, 1654, 1459, 1380, 1126 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 5.65-5.57 (m, 1H, H-2), 4.32 (t, 1H, *J* = 5.7 Hz, H-8), 4.08 (d, 2H, *J* = 6.0 Hz, H-1), 3.29 (s, 6H, 2 X $OC\underline{H}_3$), 2.05 (dd, 1H, *J* = 14.0, 5.4 Hz, 1 X H-4), 1.89 (dd, 1H, *J* = 14.0, 7.5 Hz, 1 X H-4), 1.70-1.42 (m, 3H, $O\underline{H}$ & H-7), 1.42-1.22 (m, 2H, H-5 & 1 X H-6), 1.22-1.08 (m, 1H, 1 X H-6), 0.87 (d, 3H, *J* = 6.6 Hz, $5\text{-}C\underline{H}_3$); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 131.1 (t, *J* = 22.5 Hz, C-3), 130.3 (C-2), 104.8 (C-8), 63.7 (C-1), 52.7 ($O\text{-}\underline{C}H_3$), 52.5 ($O\text{-}\underline{C}H_3$), 39.5 (C-4), 32.8 (C-5), 31.1 ($\underline{C}H_2$), 30.0 ($\underline{C}H_2$), 19.4 ($5\text{-}\underline{C}H_3$); HRMS (ES) calcd for $C_{11}H_{21}DO_3$ 203.1631, found 226.1526 $[M+Na]^+$.

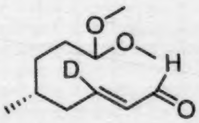
(2*E*,5*R*)-8,8-Dimethoxy-5-methyloct-2-enal-d_1 (93) (Method a)



The same method as for the preparation of **102** was employed. Thus, reaction of **92** (1.7494 g, 8.61 mmol) with DMP (4.78 g, 11.27 mmol) afforded **93** (1.6677 g, 96%) after purification by flash chromatography (SiO_2, 1:1/Et_2O:pentane, R_f 0.41). $[\alpha]_D^{20}$ +14.65° (*c* 1.07, $CHCl_3$); IR (microscope) 2956, 2832, 2681, 1704, 1637 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 9.49 (d, 1H, *J* = 7.8 Hz, H-1), 6.12-6.05 (m, 1H, H-2), 4.31 (t, 1H, *J* = 5.7 Hz, H-8), 3.29 (s, 6H, 2 X $OC\underline{H}_3$), 2.33 (ddd, 1H, *J* = 14.4, 5.5, 0.6 Hz, 1 X H-4), 2.16 (ddd, 1H, *J* = 14.4, 7.5, 0.6 Hz, 1 X H-4), 1.74-

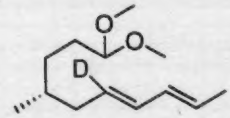
1.48 (m, 3H, H-5 & H-7), 1.46-1.32 (m, 1H, 1 X H-6), 1.30-1.12 (m, 1H, 1 X H-6), 0.92 (d, 3H, *J* = 6.6 Hz, 5-C$\underline{H}_3$); ^{13}C NMR (CDCl$_3$, 125 MHz) δ 193.9 (C-1), 156.4 (C-3), 134.2 (C-2), 104.6 (C-8), 52.9 (O-$\underline{C}$H$_3$), 52.7 (O-$\underline{C}$H$_3$), 39.9 (C-4), 32.4 (C-5), 31.3 ($\underline{C}$H$_2$), 30.0 ($\underline{C}$H$_2$), 19.5 (5-$\underline{C}$H$_3$); HRMS (EI) calcd for $C_{11}H_{19}DO_3$ 201.1474, found 200.1395 [M-H]$^+$, 170.1284 [M-OCH$_3$]$^+$.

(2*E*,5*R*)-8,8-Dimethoxy-5-methyloct-2-enal-*d*$_1$ (93) (Method b)



TPAP (7 mg, 19.9 μmol) and NMO (232 mg, 1.98 mmol) were added sequentially to a stirred solution of **92** (365.6 mg, 1.80 mmol) in dry CH$_2$Cl$_2$ at rt and left to react for 1.5 days. The solvent was evaporated under reduced pressure and the mixture purified by flash chromatography (2 columns) (SiO$_2$, 1:1/Et$_2$O:pentane, R$_f$ 0.41) to yield **93** (179.7 mg, 50%). The compound had the same spectroscopic properties as **93** produced by Method a.

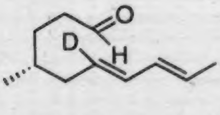
(2*E*,4*E*,7*R*)-10,10-Dimethoxy-7-methyldeca-2,4-diene-*d*$_1$ (94)



Compound **94** was prepared using a modified version of the method described by Schlosser and Christmann.[126] *n*-BuLi (2.5 M in hexanes) (3.65 mL, 9.11 mmol) was added to a solution of (ethyl)triphenylphosphonium iodide (3.4654g, 8.29 mmol) in Et$_2$O (50 mL) and stirred for 15 min before cooling the reaction mixture to -78 °C. To this, a solution of the aldehyde **93** (1.6677 g, 8.29 mmol) in Et$_2$O (15 mL) was added dropwise over 20 min. After reacting for 15 min a second equivalent (3.65 mL, 9.11 mmol) of 2.5 M nBuLi in hexanes was added followed by a solution of ethereal HCl (1.18 M, 7.72 mL, 9.11 mmol). After 5 minutes a solution of *t*-

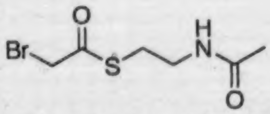
BuOK (2.045 g, 18.23 mmol) in *t*-BuOH (20 mL) was added and the reaction mixture left to warm to rt over 2 h. The reaction mixture was then extracted with H_2O (4 X 30 mL) and washed with brine. The aqueous fractions were combined and back extracted with Et_2O (2 X 50 mL). The organic fractions were combined, dried ($MgSO_4$), filtered and the solvents evaporated under reduced pressure. The resulting mixture was purified by flash chromatography (SiO_2, 5% Et_2O in pentane) to give a 1:1 mixture of *E*:*Z* isomers by ^{1}H-NMR. This mixture was further purified by flash chromatography ($AgNO_3$ impregnated SiO_2 (2 columns) using 5% Et_2O in pentane, R_f 0.25) to give the pure *E*,*E* **94** compound (0.2902 g, 16%). $[\alpha]^{20}_{D}$ -1.93° (*c* 1.98, $CHCl_3$); IR (microscope) 2953, 2929, 2829, 2225, 1458, 1378, 1127, 1058 cm^{-1}; ^{1}H NMR (CD_2Cl_2, 300 MHz) δ 6.10-5.94 (m, 2H, H-3 & H-4), 5.56 (ddq, 1H, *J* = 13.8, 6.9, 0.5 Hz, H-2), 4.28 (t, 1H, *J* = 5.7 Hz, H-10), 3.27 (s, 6H, 2 X $OC\underline{H}_3$), 2.06 (dd, 1H, *J* = 13.8, 5.4 Hz, 1 X H-6), 1.90 (dd, 1H, *J* = 13.8, 7.2 Hz, 1 X H-6), 1.72 (d, 3H, *J* = 6.9 Hz, H-1), 1.67-1.42 (m, 3H, H-7 & H-9), 1.42-1.27 (m, 1H, 1 X H-8), 1.20-1.05 (m, 1H, 1 X H-8), 0.87 (d, 3H, *J* = 6.6 Hz, 7-$C\underline{H}_3$); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 131.6 (C-3), 131.5 (C-4), 129.8 (t, *J* = 22.6 Hz, C-5), 126.8 (C-2), 104.8 (C-10), 52.6 (O-$\underline{C}H_3$), 52.5 (O-$\underline{C}H_3$), 39.8 (C-6), 33.2 (C-7), 31.2 ($\underline{C}H_2$), 30.0 ($\underline{C}H_2$), 19.4 (7-$\underline{C}H_3$), 17.9 (C-1); HRMS (EI) calcd for $C_{13}H_{23}DO_2$ 213.1838, found 213.1828 $[M]^+$.

(4*R*,6*E*,8*E*)-4-Methyldeca-6,8-dienal-*d*$_1$ (95)



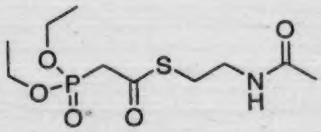
Compound **95** was prepared using a modified version of the method described by Roush and Hall.[154] A sat'd aqueous solution of oxalic acid (4 mL) was added to a solution of the acetal **94** (72 mg, 0.34 mmol) in dry THF (9 mL) and the reaction mixture was allowed to stir at rt overnight. The reaction was not complete by TLC so another 5 mL of the oxalic acid solution was added and the reaction mixture was stirred for another 24 h. The reaction mixture was then diluted with H_2O (25 mL) and Et_2O (50 mL) and the layers separated. The aqueous layer was then extracted with Et_2O (3 X 50 mL), the ethereal layers combined and washed sequentially with 5% $NaHCO_3$ (50 mL), H_2O (50 mL) and brine (50 mL). The aqueous layers were combined and back extracted with Et_2O (50 mL). All Et_2O fractions were combined, dried ($MgSO_4$), filtered and the Et_2O distilled at atmospheric pressure. The resulting oil was then purified by flash chromatography (SiO_2, 1:9/Et_2O:pentane, R_f 0.34) to yield **95** (53.8 mg, 95%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 9.75 (t, 1H, *J* = 2.1 Hz, H-1), 6.06-5.92 (m, 2H, H-7 & H-8), 5.56 (ddq, 1H, *J* = 13.2, 6.6, 1.1 Hz, H-9), 2.46-2.36 (m, 2H, H-2), 2.04 (dd, 1H, *J* = 14.0, 5.7 Hz, 1 X H-5), 1.91 (dd, 1H, *J* = 14.0, 6.3 Hz, 1 X H-5), 1.71 (d, 3H, *J* = 6.6 Hz, H-10), 1.55-1.20 (m, 3H, H-3 & H-4), 0.85 (d, 3H, *J* = 6.6 Hz, 4-C$\underline{H}_3$); HRMS (EI) calcd for $C_{11}H_{17}DO$ 167.1419, found 167.1418 $[M]^+$.

N-Acetyl-*S*-(2-bromoacetyl)cysteamine thioester (96)



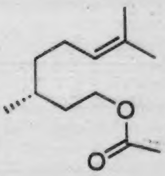
The known compound **96**[155] was prepared using the method of Roblot *et al.*[155] A solution of N-acetylcysteamine (4.061 g, 34.1 mmol) in dry THF (20 mL) was added to a stirred, cooled (0 °C) solution of bromoacetyl bromide (6.877 g, 34.0 mmol) in dry THF (30 mL). The reaction mixture was warmed to rt for 1 h before cooling to (0 °C) and sat'd $NaHCO_3$ (30 mL) was added. The THF was removed under reduced pressure and the aqueous layer extracted with CH_2Cl_2 (3 X 100 mL). The organic layers were combined, dried ($MgSO_4$), filtered and the solvent removed under reduced pressure. The mixture was purified by flash chromatography (SiO_2, EtOAc) to yield **96** as a white solid upon freezing (1.2946 g, 16%). (SiO_2, 5% MeOH in EtOAc, R_f 0.45). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 5.80 (br s, 1H, NH), 4.02 (s, 2H, CH_2Br), 5.46 (dt, 2H, J = 6.3, 6.3 Hz, CH_2CH_2NH), 3.09 (t, 2H, J = 6.3 Hz, SCH_2CH_2), 1.97 (s, 3H, $C(O)CH_3$); HRMS (EI) calcd for $C_6H_{10}BrNO_2S$ 238.9616, 240.9595 found 261.9505 $[M+Na]^+$, 263.9492 $[M+Na]^+$.

S-[2-(Acetylamino)ethyl] (diethoxyphosphoryl)ethanethioate (97)



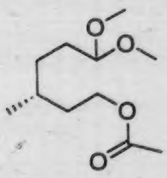
The known compound **97**[124] was prepared using the method described by Van den Heever.[124] Triethyl phosphite (0.393 mL, 2.29 mmol) was added to a solution of the bromide **96** (501 mg, 2.1 mmol) in dry toluene (15 mL) and the mixture refluxed for 4.5 days. The mixture was cooled to rt and the solvent was removed under reduced pressure to yield a yellowish brown oil. The oil was flushed through a silica plug (SiO_2, 1:1:0.1/MeCN:EtOAc:MeOH) to remove impurities. The solvent was removed under reduced pressure to give an oil that was recrystallized from pentane to yield **97** (305 mg, 49%) as white crystals. IR (microscope) 3293, 3078, 2984, 2932, 1678, 1552, 1254, 1024 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 6.25-6.05 (br s, 1H, NH), 4.15 (ddq, 4H, J = 7.5, 6.9, 0.6 Hz, 2 X $P(O)OCH_2CH_3$), 3.45 (dt, 2H, J = 6.3, 6.3 Hz, SCH_2CH_2NH), 3.22 (d, 2H, J = 21.3 Hz, $P(O)CH_2C(O)$), 3.07 (t, 2H, J = 6.3 Hz, SCH_2CH_2NH), 1.96 (s, 3H, $C(O)CH_3$), 1.34 (dt, 6H, J = 7.5, 0.6 Hz, 2 X $P(O)OCH_2CH_3$); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 190.5 (C(O)), 170.5 (C(O)), 62.9 (2 X $P(O)OCH_2CH_3$), 43.0 (d, J = 131.4 Hz, $P(O)CH_2C(O)$), 39.0 ($S\underline{C}H_2CH_2NH$), 29.5 ($SCH_2\underline{C}H_2NH$), 23.0 ($C(O)\underline{C}H_3$), 16.3 (2 X $P(O)OCH_2\underline{C}H_3$); HRMS (ES) calcd for $C_{10}H_{20}NO_5PS$ 297.0799, found 297.0794 $[M]^+$.

(3*R*)-3,7-Dimethyloct-6-en-1-yl acetate (99)



The known compound **99**[73,156] was prepared using a modified version of the method described by Stork *et al.*[156] Acetyl chloride (7.82 mL, 109.55 mmol) was added dropwise to a cooled (0 °C) solution of *R*-(+)-β-citronellol (4.28 g, 27.39 mmol) in dry THF (100 mL). After 5 min diisopropylamine (15.74 mL, 112.30 mmol) was added dropwise and the reaction mixture was allowed to stir and warm to rt for 8 h. The reaction mixture was filtered and the solid washed well with Et_2O. The solvent was evaporated under reduced pressure to give an oil which was purified by flash chromatography (SiO_2, 1:1 Et_2O:pentane, R_f 0.92) to yield **99** (5.1030 g, 94%) as a colourless oil. $[\alpha]^{20}_{D}$ +2.96° (*c* 2.42, $CHCl_3$) (lit.[156] $[\alpha]^{20}_{D}$ +5.12° (*c* 5.86, $CHCl_3$)); IR (microscope) 2964, 2917, 1742, 1236 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 5.06 (m, 1H, H-6), 4.14-4.01 (m, 2H, H-1), 2.02 (s, 3H, H-2′), 2.04-1.90 (m, 2H, H-5), 1.70-1.58 (m, 1H, 1 X H-2), 1.66 (s, 3H, $HC{=}CC\underline{H}_3$), 1.58 (s, 3H, $HC{=}CC\underline{H}_3$), 1.58-1.49 (m, 1H, H-3), 1.47-1.39 (m, 1H, 1 X H-2), 1.38-1.26 (m, 1H, 1 X H-4), 1.22-1.10 (m, 1H, 1 X H-4), 0.89 (d, 3H, $J = 6.6$ Hz, $3\text{-}C\underline{H}_3$); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 171.3 (C-1′), 131.4 (C-7), 124.6 (C-6), 63.1 (C-1), 37.0 (C-2), 35.5 (C-4), 29.5 (C-3), 25.7 ($7\text{-}\underline{C}H_3$), 25.4 (C-5), 21.1 (C-2′), 19.5 ($3\text{-}\underline{C}H_3$), 17.7 ($7\text{-}\underline{C}H_3$); HRMS (EI) calcd for $C_{12}H_{22}O_2$ 198.1620, found $[M]^+$.

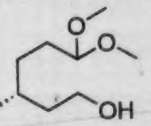
(3*R*)-6,6-Dimethoxy-3-methylhexyl acetate (100)



The known compound **100**[73,124] was prepared using the method described by Van den Heever.[124] In a 3-neck flask, a solution of **99** (5.0798 g, 25.60 mmol) in dry 1:1/CH_2Cl_2:MeOH (60 mL total volume) was cooled to -78 °C and ozonalized for 40 min (until the blue colour remained). The ozone was turned off and the reaction mixture flushed with argon for 35 min. *p*-TsOH (487 mg, 2.56 mmol) was added and the reaction mixture warmed to rt over 1.5 h. Me_2S (3.76 mL, 51.23 mmol) was added slowly over 30 min and the reaction mixture was allowed to stir for 2 h before transferring the mixture to a round bottom flask and half of the solvent evaporated under reduced pressure. The resulting solution was then diluted with H_2O and CH_2Cl_2, the layers separated and the aqueous extracted with CH_2Cl_2 (3 X 30 mL). The organic fractions were combined and washed with H_2O. The aqueous layer was then back extracted with CH_2Cl_2 and the organic fractions combined, dried ($MgSO_4$), filtered and the solvent evaporated under reduced pressure. The crude product was purified by flash chromatography (SiO_2, 1:2/Et_2O:pentane). The mixed fractions that were obtained were further purified by 2 flash chromatography columns (SiO_2, 1:4/Et_2O:pentane) (R_f 0.46 on SiO_2, 1:1/Et_2O:pentane) to yield **100** (4.4045 g, 79%). $[\alpha]_D^{20}$ +3.26° (*c* 2.75, $CHCl_3$) (lit.[73] $[\alpha]_D^{20}$ +2.23° (*c* 2.38, $CHCl_3$)); IR (microscope) 2960, 2932, 2723, 1738, 1461, 1368, 1243 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 4.32 (t, 1H, *J* = 5.7 Hz, H-6), 4.13-4.04 (m, 2H, H-1), 3.30 (s, 6H, 2 X $OC\underline{H}_3$), 2.03 (s, 3H, H-2′), 1.70-1.50 (m, 4H, 1 X H-2, & H-3, & H-5), 1.49-1.32 (m, 2H, 1 X H-2, & 1 X H-4), 1.24-1.14 (m, 1H, 1 X H-4), 0.90 (d, 3H, *J* = 6.3 Hz, 3-CH_3); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 170.9 (C-1′), 104.7 (C-6), 62.7 (C-1), 52.4 ($O\underline{C}H_3$), 52.3 ($O\underline{C}H_3$), 35.2 ($\underline{C}H_2$), 31.5 ($\underline{C}H_2$), 29.8 ($\underline{C}H_2$), 29.6 (C-3), 20.8

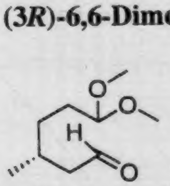
(C-2), 19.3 (3-$\underline{C}H_3$); HRMS (EI) calcd for $C_{11}H_{22}O_4$ 218.1518, found 217.1440 $[M\text{-}H]^+$, 187.1333 $[M\text{-}OCH_3]^+$.

(3*R*)-6,6-Dimethoxy-3-methylhexan-1-ol (101)



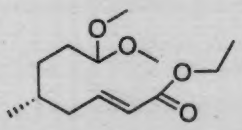
The known compound **101**[73,124] was prepared using the method described by Witter.[73] NaOMe (155 mg, 2.87 mmol) was added to a stirred solution of **100** (1.5455 g, 7.08 mmol) in dry MeOH (20 mL). After 24 h, the reaction mixture was diluted with H_2O (6 mL) and allowed to stir for 30 min before most of the solvent was evaporated under reduced pressure. The remainder was diluted with Et_2O and washed with H_2O (3 X 5 mL) and brine (2 X 5 mL). The aqueous layers were combined and back extracted with Et_2O. The ethereal layers were combined, dried ($MgSO_4$), filtered and the solvent evaporated under reduced pressure to yield **101** (1.2065 g, 97%) as a colourless oil that was used without further purification. (R_f 0.13 on SiO_2, 1:1/Et_2O:pentane). $[\alpha]_D^{20}$ +8.30° (*c* 1.13, $CHCl_3$) (lit.[73] $[\alpha]_D^{20}$ +2.75° (*c* 2.22, $CHCl_3$)); IR (microscope) 3458 (br), 2952, 2929, 2874, 2830, 1459, 1380, 1126, 1061 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 4.26 (t, 1H, J = 5.7 Hz, H-6), 3.57-3.40 (m, 2H, H-1), 3.13 (s, 6H, 2 $XOC\underline{H}_3$), 1.70-1.49 (m, 4H, 1 X H-2, & H-3, & H-5), 1.49-1.32 (m, 1H, 1 X H-2), 1.32-1.12 (m, 2H, H-4), 0.83 (d, 3H, J = 6.6 Hz, 3-$C\underline{H}_3$); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 104.8 (C-6), 60.0 (C-1), 52.6 ($O\underline{C}H_3$), 52.5 ($O\underline{C}H_3$), 39.7 ($\underline{C}H_2$), 31.6 ($\underline{C}H_2$), 29.9 ($\underline{C}H_2$), 29.7 (C-3), 19.5 (3-$\underline{C}H_3$); HRMS (EI) calcd for $C_9H_{20}O_3$ 176.1412, found 175.1334 $[M\text{-}H]^+$, 145.1228 $[M\text{-}OCH_3]^+$.

(3*R*)-6,6-Dimethoxy-3-methylhexanal (102)



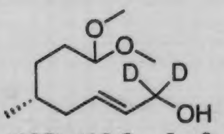
The known compound **102**[73,124] was prepared using a modification of the method described by Witter.[73] A solution of **101** (1.2065 g, 6.80 mmol) in dry CH_2Cl_2 (10 mL) was added to a stirred suspension of DMP (3.48 g, 8.21 mmol) in dry CH_2Cl_2 (10 mL) and stirred at rt for 1.5 days. The reaction mixture was diluted with Et_2O and 1.3 M NaOH (40 mL) and stirred for 30 min The two layers were then separated and the organic layer washed sequentially with 1.3 M NaOH and H_2O. The organic layer was dried ($MgSO_4$), filtered and the solvent evaporated under reduced pressure to yield an oil which was purified by flash chromatography (SiO_2, 1:5/Et_2O:pentane) to yield **101** (829.4 mg, 70%) (R_f 0.44 on SiO_2, 1:1/Et2O:pentane). $[\alpha]_D^{20}$ +12.34° (*c* 0.41, $CHCl_3$) (lit.[73] $[\alpha]_D^{20}$ +13.23° (*c* 1.41, $CHCl_3$)); IR (microscope) 2954, 2930, 2874, 2832, 2720, 1725, 1459, 1381, 1128 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 9.71, (t, 1H, *J* = 2.3 Hz, H-1), 4.30 (t, 1H, *J* = 5.4 Hz, H-6), 3.27 (s, 6H, 2 X $OC\underline{H}_3$), 2.38 (ddd, 1H, *J* = 16.2, 5.7, 2.3 Hz, 1 X H-2), 2.21 (ddd, 1H, *J* = 16.2, 7.8, 2.3 Hz, 1 X H-2), 2.04 (m, 1H, H-3), 1.71-1.48 (m, 2H, H-5), 1.41-1.12 (m, 2H, H-4), 0.96 (d, 3H, *J* = 6.6 Hz, 3-$C\underline{H}_3$); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 202.9 (C-1), 104.6 (C-6), 52.8 (1 X $O\underline{C}H_3$), 52.8 (1 X $O\underline{C}H_3$), 51.0 (C-2), 31.6 ($\underline{C}H_2$), 30.0 ($\underline{C}H_2$), 27.9 (C-3), 19.8 (3-$\underline{C}H_3$); HRMS (EI) calcd for $C_9H_{18}O_3$ 174.1256, found 173.1334 $[M-H]^+$, 145.1228 $[M-OCH_3]^+$.

Ethyl (2*E*,5*R*)-8,8-dimethoxy-5-methyloct-2-enoate (103)



The known compound **103**[73,124] was prepared using a modified version of the method described by Seebach and coworkers.[73,150] A solution of the aldehyde **102** (1.755 g, 10.10 mmol) in dry THF (50 mL) was added to a cooled (0 °C) stirred solution of (carbethoxymethylene)triphenylphosphorane (4.75 g, 11.11 mmol) in dry THF (40 mL). The reaction mixture was allowed to stir and warm to rt overnight at which time it was heated to 40 °C for 24 h before washing with H_2O (twice) and brine. The aqueous layers were combined and back extracted with THF, the organic fractions were combined, dried ($MgSO_4$), filtered and the solvent evaporated under reduced pressure. The resulting mixture was purified by flash chromatography (3 columns, each, SiO_2, 1:2/Et_2O:pentane) to yield **103** (1.6881 g, 68%) (R_f 0.64 on SiO_2, 1:1/Et2O:pentane). $[\alpha]_D^{20}$ 3.10° (*c* 0.78, $CHCl_3$) (lit.[73] $[\alpha]_D^{20}$ +2.98° (*c* 1.98, $CHCl_3$)); IR (microscope) 2954, 2830, 1721, 1654, 1461, 1368, 1315, 1267, 1187, 1127, 1051 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 6.90 (dt, 1H, *J* = 15.4, 8.0 Hz, H-3), 5.79 (dt, 1H, *J* = 15.4, 1.5 Hz, H-2), 4.32 (t, 1H, *J* = 5.7 Hz, H-8), 4.16 (q, 2H, *J* = 7.2 Hz, H-1′), 3.29 (s, 6H, 2 X $OC\underline{H}_3$), 2.18 (dddd, 1H, *J* = 14.1, 8.0, 5.4, 1.5 Hz, 1 X H-4), 2.04 (dddd, 1H, *J* = 14.1, 8.4, 8.0, 1.5 Hz, 1 X H-4), 1.70-1.45 (m, 3H, 1 X H-6 & H-7), 1.45-1.30 (m, 1H, H-5), 1.27 (t, 3H, *J* = 7.2 Hz, H-2′), 1.25-1.12 (m, 1H, 1 X H-6), 0.90 (d, 3H, *J* = 6.6 Hz, 5-$C\underline{H}_3$); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 166.5 (C-1), 147.7 (C-3), 122.6 (C-2), 104.7 (C-8), 60.1 (C-1′), 52.7 (1 X $O\underline{C}H_3$), 52.6 (1 X $O\underline{C}H_3$), 39.5 (C-4), 32.4 (C-5), 31.3 ($\underline{C}H_2$), 30.0 ($\underline{C}H_2$), 19.4 (5-$\underline{C}H_3$), 14.2 (C-2′); HRMS (ES) calcd for $C_{13}H_{24}O_4$ 244.1675, found 267.1567 $[M+Na]^+$.

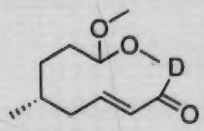
(2*E*,5*R*)-8,8-Dimethoxy-5-methyloct-2-en-1-ol-*d*$_2$ (104)



Compound **104** was prepared using a modified version of the method described by Nicolaou *et al.*[153] An ethereal solution of DIBAL-D (**108**) (18.0 mL, 0.45 M) was added to a cooled (-78 °C) solution of **103** (1.7016 g, 6.96

mmol) in dry THF (15 mL) dropwise over 30 min and the reaction mixture was allowed to stir and warm to rt overnight. Sat'd $NaHCO_3$ (10 mL) was added to the cooled (0 °C) reaction mixture and the aluminum salts removed by filtration through a pad of celite. The celite washed with EtOAc, and the solvents were evaporated under reduced pressure and the crude mixture purified by flash chromatography (SiO_2, 1:1/Et_2O:pentane, R_f 0.19) to yield **92** (0.281 g, 19.7%). ^1H NMR ($CDCl_3$, 300 MHz) δ 5.67-5.44 (m, 2H, H-2 & H-3), 4.29 (t, 1H, J = 5.7 Hz, H-8), 3.26 (s, 6H, 2 X $OC\underline{H}_3$), 2.10-1.95 (m, 1H, 1 X H-4), 1.92-1.78 (m, 1H, 1 X H-4), 1.67-1.42 (m, 3H, H5 & H-7), 1.42-1.24 (m, 1H, 1 X H-6), 1.20-1.03 (m, 1H, 1 X H-6), 0.84 (d, 3H, J = 6.6 Hz, 5-$C\underline{H}_3$); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 131.2 (C-3), 130.3 (C-2), 104.7 (C-8), 62.8 (qn, J = 2.5 Hz, C-1), 52.6 (O-$\underline{C}H_3$), 52.4 (O-$\underline{C}H_3$), 39.5 (C-4), 32.7 (C-5), 31.0 ($\underline{C}H_2$), 29.9 ($\underline{C}H_2$), 19.3 (5-$\underline{C}H_3$); HRMS (ES) calcd for $C_{11}H_{20}D_2O_3$ 204.1692, found 227.1588 $[M+Na]^+$.

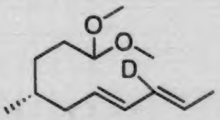
(2*E*,5*R*)-8,8-Dimethoxy-5-methyloct-2-enal-d_1 (105)



105 Was prepared by Dr. Belén Mayo-Martín[128] using the same method as for the preparation of **102**. DMP (3.0 g, 7.1 mmol) was added slowly to a cooled (0 °C) solution of **104** (420 mg, 2.1 mmol) in distilled CH_2Cl_2 (50 mL). The reaction mixture was allowed to stir and warm to rt for 2 h. The reaction was quenched by the addition Et_2O (50 mL) followed by aqueous NaOH solution (50 mL, 1.3 M). The mixture was stirred for 1 h. The organic layer was separated and washed with NaOH (100 mL, 1.3 M) and H_2O (50 mL). The organic layer was dried (Na_2SO_4), filtered and the solvent evaporated under reduced pressure to give a colourless oil which was purified by flash chromatography (SiO_2, 1:1/Et_2O:pentane, R_f 0.35) to yield **105** as a

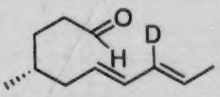
colourless oil (330 mg, 80%). $[\alpha]^{27}_{\mathrm{D}}$ –0.72° (*c* 0.86, CH_2Cl_2); ^{1}H NMR ($CDCl_3$, 300 MHz,) δ 6.84-6.70 (m, 1H, H-3), 6.07 (d, 1H, *J* = 15.6 Hz, H-2), 4.29 (t, 1H, *J* = 5.6 Hz, H-8), 3.27 (s, 6H, 2 × $OC\underline{H}_3$), 2.37-2.24 (m, 1H, 1 X H-4), 2.22-2.08 (m, 1H, 1 X H-4), 1.72-1.44 (m, 3H, H-5 & H-7), 1.44-1.10 (m, 2H, H-6), 0.90 (d, 3H, *J* = 6.6 Hz, 5-$C\underline{H}_3$); ^{13}C NMR (125 MHz, $CDCl_3$) δ 193.3 (d, *J* = 26 Hz, C-1), 156.9 (C-3), 134.2 (C-2), 104.5 (C-8), 52.7 (1 X $O\underline{C}H_3$), 52.6 (1 X $O\underline{C}H_3$), 40.0 (C-4), 32.4 (C-5), 31.2 (C-6), 30.0 (C-7), 19.4 (5-$\underline{C}H_3$); HRMS (ES) calcd $C_{11}H_{19}DO_3$ 201.1474, found 224.1368 $[M+Na]^+$.

(2*E*,4*E*,7*R*)-10,10-Dimethoxy-7-methyldeca-2,4-diene-*d*$_1$ (106)



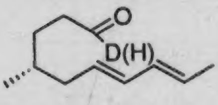
106 was prepared by Dr. Belén Mayo-Martín[128] using the same method used for the preparation of **94**. *n*-BuLi (2.5 M in hexane, 4.3 mL) was added to the suspension of ethyl triphenylphosphonium iodide (690 mg, 1.65 mmol) in dry THF (6 mL) and dry Et_2O (6 mL), the mixture turned yellow, then orange, then red, then bright red. The mixture was stirred for 10 min at rt before cooling to –78 °C (colour became orange). A solution of **105** (330 mg, 1.65 mmol) in freshly distilled Et_2O (5 mL) was slowly added to the phosphorane solution. The reaction mixture was stirred for 25 min at –78 °C (colour: yellow to brown). *n*-BuLi (2.5 M in hexane, 0.73 mL, 1.81 mmol) was added to the reaction (colour: brown-red then brown). The mixture was stirred for 5 min at –78 °C and an ethereal HCl solution (1.53 mL, 1.18 M HCl in Et_2O, 1.81 mmol) was added, followed by the addition of potassium *tert*-butoxide (428 g, 3.63 mmol) in *tert*-BuOH (3 mL). The mixture was allowed to stir and warm to rt for 1 h. The mixture was diluted with Et_2O (50 mL) and washed with H_2O (3 X 30 mL) and brine (20 mL). Then the organic layer was dried (Na_2SO_4), filtered and the solvent evaporated under reduced pressure to give an orange liquid. The crude product was purified by flash chromatography (SiO_2, 5% Et_2O in pentane, R_f 0.24) to yield **106** as a colourless oil (290 mg, 82%). $[\alpha]_D^{20}$ –3.10° (c 0.40 in CH_2Cl_2); IR ($CHCl_3$ cast) 2952 (s) cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 5.99 (d, 1H, J = 15.3 Hz, H-4), 5.62-5.46 (m, 2H, H-5 & H-2), 4.34 (t, 1H, J = 5.7 Hz, H-10), 3.31 (s, 6H, 2 X $OC\underline{H}_3$), 2.20-1.84 (m, 2H, H-6), 1.73 (d, 3H, J = 6.6 Hz, H-1), 1.70-1.08 (m, 5H, H-7 & H-8 & H-9), 0.88 (d, 3H, J = 6.6 Hz, 7-$C\underline{H}_3$); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 131.6 ($\underline{C}H$=CH), 131.3 (d, J = 23 Hz, C-3), 130.1 ($\underline{C}H$=CH), 126.6 ($\underline{C}H$=CH), 104.8 (C-10), 52.5 (1 X $O\underline{C}H_3$), 52.4 (1 X $O\underline{C}H_3$), 39.9 (C-6), 33.2 (C-7), 31.2 (C-8), 30.1 (C-9), 19.4 (7-$\underline{C}H_3$), 17.8 (C-1); HRMS (ES) calcd $C_{13}H_{23}DO_2$ 213.1838, found 236.17300 $[M+Na]^+$.

(4*R*,6*E*,8*E*)-4-Methyldeca-6,8-dienal-*d*$_1$ (107)



107 Was prepared by Dr. Belén Mayo-Martín[128] using the same method used for the preparation of **95**. Oxalic acid (fresh saturated aqueous solution, 12 mL) was added to the stirred solution of **106** (170 mg, 0.80 mmol) dissolved in THF (8 mL, ACS grade) and the reaction mixture stirred at rt overnight. The mixture was diluted with Et_2O (50 mL) and H_2O (30 mL). The aqueous layer was separated and extracted with Et_2O (3 × 30 mL). The combined ethereal extracts were washed with saturated aqueous $NaHCO_3$ (2 × 20 mL) and H_2O (1 × 30 mL). The organic layer was dried (Na_2SO_4), filtered and the solvent evaporated under reduced pressure to give a colourless liquid which was purified by flash column chromatography (SiO_2, 5% Et_2O in pentane, R_f 0.25) to give a colourless oil (80 mg, 60%). ^1H NMR ($CDCl_3$, 500 MHz) δ 9.74 (t, 1H, J = 1.8 Hz, H-4), 6.04-5.94 (m, 1H, $C\underline{H}$=CH), 5.62-5.52 (m, 1H, CH=CH), 5.52-5.44 (m, 1H, CH=CH), 2.48-2.34 (m, 2H, H-2), 2.10-2.00 (m, 1H, 1 X H-5), 1.96-1.88 (m, 1H, 1 X H-5), 1.71 (d, 3H, *J* = 6.3 Hz, H-10), 1.74-1.62 (m, 1H, H-4), 1.57–1.38 (m, 2H, H-3), 0.87 (d, 3H, *J* = 6.7 Hz, 4-CH$_3$); HRMS (ES) calcd $C_{11}H_{17}DO$ 167.1419, found 190.13147 [M+Na]$^+$.

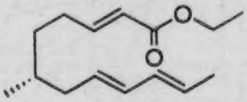
(4*R*,6*E*,8*E*)-4-Methyldeca-6,8-dienal-*d*$_1$ (111)



A solution of the aldehyde **109** (62 mg, 0.37 mmol) in distilled Et_2O (4 mL) was added to a stirred, cooled (0 °C) solution of lithium aluminium deuteride (180 mL, 1.0 M in Et_2O, 0.188 mmol) in distilled Et_2O (8 mL). The reaction mixture was stirred for 1.5 h and allowed to warm to rt. The reaction mixture was cooled to 0 °C and diluted with Et_2O (15 mL). H_2O (1.5 mL) was slowly added to the mixture. The reaction mixture was stirred for 30 min at 0 °C before the mixture was filtered through a layer of celite. The filtrate was dried (Na_2SO_4), filtered and solvent evaporated under reduced pressure to give a colourless oil, which was purified by flash chromatography (SiO_2, 40% Et_2O in pentane, R_f 0.23) to yield the alcohol **110** (55 mg, 86%) as a colourless oil. DMP (496 mg, 1.17 mmol) was added to a stirred, cooled (0 °C) solution of the alcohol (40 mg, 0.24 mmol) in distilled CH_2Cl_2 (10 mL) and the reaction mixture stirred and allowed to warm to rt for 3 h. The reaction mixture was diluted with Et_2O (12 mL) and quenched with NaOH (10 mL, 1.3 M). The mixture was stirred for 1 h and the layers separated. The ethereal layer was washed with NaOH (10 mL, 1.3 M) and H_2O (10 mL). The organic layer was dried (Na_2SO_4), filtered and the solvent evaporated under reduced pressure to give a colourless oil that was purified by flash chromatography (SiO_2, 5% Et_2O in pentane, R_f 0.30) to yield **111** (30 mg, 77%) as a colourless oil. The ratio of non-deuterated:deuterated aldehyde was 40: 60. $[\alpha]_D^{20}$ –4.21 (*c* 0.19, CH_2Cl_2); IR

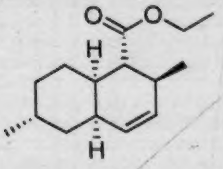
(oil) 3016 (m), 1716 (s) cm^{-1}; ^{1}H NMR (CDCl$_3$, 400 MHz) δ 6.05-5.91 (m, 2H, H-7 & H-8), 5.62-5.42 (m, 2H, H-6 & H-9), 2.47-2.32 (m, 2H, H-2), 2.10-1.98 (m, 1H, 1 X H-5), 1.96-1.85 (m, 1H, 1 X H-5), 1.70 (d, 3H, *J* = 6.8 Hz, H-10), 1.73-1.61 (m, 1H, H-4), 1.55-1.30 (m, 2H, H-3), 0.86 (d, 3H, *J* = 6.6 Hz, 4-C$\underline{H}_3$); HRMS (ES) calcd $C_{11}H_{17}DO$ 167.1419, found 190.1311 [M+Na]$^+$.

Ethyl (2*E*,6*R*,8*E*,10*E*)-6-methyldodeca-2,8,10-trienoate (112)



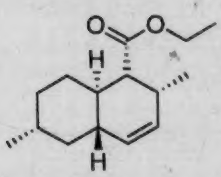
The known compound **112**[73] was prepared using a modified version of the method of Seebach and coworkers.[150] A solution of the aldehyde **109** (6.4 mg, 38 μmol) in dry CH_2Cl_2 (800 μL) was added to a cooled (0 °C) stirred solution of (carbethoxymethylene)triphenylphosphorane (26 mg, 60 μmol) in dry CH_2Cl_2 (1 mL). The reaction mixture was stirred for 30 min before warming to rt for 5.5 h. The solvent was then evaporated under reduced pressure and purified by flash chromatography (SiO$_2$, 1:9/Et$_2$O:pentane, R_f 0.58) to yield pure **112** (1.6 mg, 18%). ^{1}H NMR (CDCl$_3$, 300 MHz) δ 6.93 (dt, 1H, *J* = 15.6, 6.9 Hz, H-3), 6.06-5.90 (m, 2H, H-9 & H-10), 5.77 (dt, 1H, *J* = 15.6, 1.5 Hz, H-2), 5.64-5.42 (m, 2H, H-8 & H-11), 4.16 (q, 2H, *J* = 7.2 Hz, H-1′), 2.30-2.09 (m, 2H, H-4), 2.09-1.97 (m, 1H, 1 X H-7), 1.97-1.84 (m, 1H, 1 X H-7), 1.71 (d, 3H, *J* = 6.6 Hz, H-12), 1.57-1.40 (m, 2H, 1 X H-5, & H-6), 1.27 (t, 3H, *J* = 7.2 Hz, H-2′), 1.34-1.17 (m, 1H, 1 X H-5), 0.86 (d, 3H, *J* = 6.6 Hz, 6-C$\underline{H}_3$); HRMS (EI) calcd for $C_{15}H_{24}O_2$ 236.1776, found 236.1768 [M]$^+$.

Ethyl (1*R*,2*S*,4a*R*,6*R*,8a*R*)-2,6-dimethyl-1,2,4a,5,6,7,8,8a-octahydronaphthalene-1-carboxylate (*exo*) (113)



The same method as for the preparation of **114** was employed. Thus, reaction of **75** (1.4 mg, 4.52 μmol) in dry EtOH (0.5 mL) with NaOEt (0.05 mL of a 0.5M sol'n) stirred for 16.5 h before working up and purification afforded **113**. GC-MS (t_r 14.34 min). A good ^{1}H-NMR could not be obtained, however a good ^{1}H-NMR was obtained of the standard prepared by Dr. David Witter,[73] and the GC-MS had the same retention time as stated above and the same mass fragmentation pattern as for **114**.

Ethyl (1*R*,2*R*,4a*S*,6*R*,8a*R*)-2,6-dimethyl-1,2,4a,5,6,7,8,8a-octahydronaphthalene-1-carboxylate (*endo*) (114)



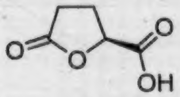
The known compound **114**[73,77] was prepared using a modified version of the method described by Auclair *et al.*[77] NaOEt (0.1 mL of a 0.5M sol'n) was added slowly to a solution of **76** (3.4 mg, 10.9 μmol) in dry EtOH (0.5 mL) and the reaction mixture stirred at rt for 16.5 h. After checking by TLC, the majority of the starting material remained, so more NaOEt (0.1 mL of a 0.5M sol'n) was added and the reaction mixture heated to 50 °C for 24 h. The reaction seemed complete by TLC and was diluted with H_2O and neutralized with 0.5 M HCl. The mixture was extracted with $CHCl_3$ (5 X 5 mL), the organic fractions were dried ($MgSO_4$), filtered and the solvent evaporated under reduced pressure to yield 1.7 mg of crude product which was purified by flash chromatography (SiO_2, 5% Et_2O in pentane, R_f 0.32) to yield **114**. GC-MS (t_r 14.25 min) had not been performed previously on this compound. ^{1}H

NMR matched the previously synthesized product.[73] ^{1}H NMR ($CDCl_3$, 600 MHz) δ 5.53 (ddd, 1H, *J* = 9.9, 4.2, 3.0 Hz, H-3), 5.30 (d, 1H, *J* = 9.9 Hz, H-4), 4.12 (q, 2H, *J* = 7.2 Hz, $OC\underline{H}_2CH_3$), 2.55-2.49 (m, 2H, H-1 & H-2), 1.93 (dddd, 1H, *J* = 12.0, 3.0, 3.0, 3.0 Hz, H-8eq), 1.75-1.65 (m, 3H, H-4a & H-7eq & H-5eq), 1.54-1.42 (m, 1H, H-6), 1.35 (dddd, 1H, *J* = 12.0, 12.0, 12.0, 3.0 Hz, H-8a), 1.24 (t, 3H, *J* = 7.2 Hz, $OCH_2C\underline{H}_3$), 1.04 (dddd, 1H, *J* = 12.0, 12.0, 12.0, 3.6 Hz, H-8ax), 1.00-0.95 (m, 1H, H-7ax), 0.90 (d, 3H, *J* = 7.2 Hz, 2-$C\underline{H}_3$), 0.88 (d, 3H, *J* = 6.6 Hz, 6-$C\underline{H}_3$), 0.77 (ddd, 1H, *J* = 12.0, 12.0, 12.0 Hz, H-5ax).

Thermal Diels-Alder of 74 and GC-MS analysis of the products

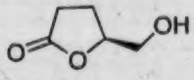
During either the enzymatic, control or thermal Diels-Alder reaction of making (**75** and **76**), followed by transesterification to the ethyl esters (**113** and **114**) and isolation of the mixture of ethyl esters by preparative TLC (SiO_2, 5% Et_2O in pentane), an additional smaller peak at (t_r 14.52 min) was seen by GC-MS which had the same mass and fragmentation pattern as (**113** and **114**). Purified **114** was added and an enhancement of the peak at (t_r 14.25 min) within the mixture occurred. Purified **113** was added and an enhancement of the peak at (t_r 14.34 min) within the mixture occurred. An authentic sample of **79** was synthesized and added to the mixture, which enhanced the peak at (t_r 14.52 min) identifying it as **79**.

(+)-(2*S*)-5-Oxotetrahydrofuran-2-carboxylic acid (116).



The known compound **116**[157,158] was prepared using the method described therein. A solution of sodium nitrite (126 g, 1.83 mmol in 270 mL H_2O) was added dropwise over 12 h to a mixture of (s)-(+)-glutamic acid sodium salt **115** (180 g, 1.06 mol in 480 mL H_2O and 252 mL conc. HCl) at 0 °C with stirring. The reaction mixture was then stirred at rt for 10 h. The solvent was evaporated under reduced pressure to give a pale yellow oil and white crystals. EtOAc (500 mL) was added and the crystals filtered off and the solvent evaporated under reduced pressure to give **116** (137.7 g, 99%) as a viscous yellow oil. For the crude, $[\alpha]_D^{20}$ +2.03° (*c* 4.67, EtOH) (lit.[157,158] $[\alpha]_D^{20}$ +15.6° (*c* 2.0, EtOH)); IR (microscope) 3500-2500 (br), 2946, 1747, 1181 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 10.0 (br s, 1H, COOH), 5.04-4.94 (m, 1H, H-2) 2.74-2.50 (m, 3H, H-3 & 1 X H-4), 2.50-2.25 (m, 1H, 1 X H-4); ^{13}C NMR ($CDCl_3$, 150 MHz) δ 176.0 (C-2), 174.5 (C-6), 75.1 (C-5), 27.0 (CH_2), 25.9 (CH_2); HRMS (EI) calcd for $C_5H_6O_4$ 130.0266, found 130.0269 $[M]^+$.

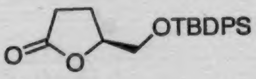
(+)-(5*S*)-5-(Hydroxymethyl)dihydrofuran-2(3*H*)-one (117)



The known compound **117**[157,158] was prepared using the method described therein. $BH_3 \bullet SMe_2$ (10 M, 128 mL, 1.28 mol) was added dropwise *via* a cannula to a cooled (0 °C) stirred solution of crude **116** (156.9 g, 1.21 mol) in dry THF (500 mL) over 5 h. The reaction mixture was allowed to stir and warm to rt overnight. Dry MeOH (200 mL) was added dropwise and the solvent evaporated under reduced pressure. The crude mixture (154.5 g) was dissolved in CH_2Cl_2, adsorbed onto SiO_2 and the solvent evaporated and the compound purified by flash chromatography (SiO_2, 30%

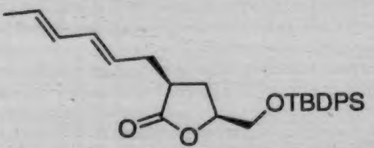
EtOAc in hexanes) to yield **117** (94.4 g, 76%). $[\alpha]_D^{20}$ +18.41° (*c* 1.55, EtOH) (lit.[157,158] $[\alpha]_D^{20}$ +31.3° (*c* 2.92, EtOH)); IR (microscope) 3403 (br), 2944, 1765, 1192, 1062 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 4.61 (ddt, 1H, *J* = 7.6, 4.8, 3.0 Hz, H-5), 3.89 (dd, 1H, *J* = 12.8, 3.0 Hz, 1 X H-6), 3.64 (dd, 1H, *J* = 12.8, 4.8 Hz, 1 X H-6), 2.66-2.48 (m, 2H, H-3), 2.30-2.20 (m, 1H, 1 X H-4), 2.18-2.08 (m, 1H, 1 X H-4), 2.06 (br s, 1H, O$\underline{H}$); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 178.0 (C-2), 81.0 (C-5), 64.0 (C-6), 28.7 ($\underline{C}H_2$), 23.1 ($\underline{C}H_2$); HRMS (EI) calcd for $C_5H_8O_3$ 116.0473, found 116.0474 $[M]^+$.

(+)-(5*S*)-5-[(*Tert*-butyldiphenylsilyloxy)methyl]dihydrofuran-2(3*H*)-one (118)



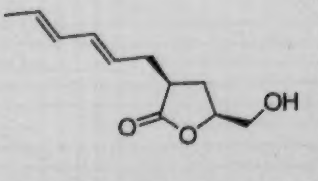
The known compound **118**[159] was prepared using the method described therein. Dried (over KOH) pyridine (132 mL, 1.63 mol) was added dropwise over 30 min to a stirred solution of **118** (47.2 g, 406 mmol) in dry CH_2Cl_2 (620 mL). To this mixture was added neat TBDPS-Cl (106 mL, 406 mmol) dropwise over 60 min and the reaction mixture left to stir for 24 h before washing the mixture with 2N HCl (2 X 200 mL) and brine (200 mL). The organics were dried ($MgSO_4$), filtered and the solvent evaporated under reduced pressure. The crude product was combined with a duplicate reaction and was recrystallized from Et_2O/petroleum Et_2O to yield **118** (164.2 g, 57% overall) of off-white crystals. $[\alpha]_D^{20}$ +33.7° (*c* 1.95, EtOH) (lit.[159] $[\alpha]_D^{20}$ +35.5° (*c* 1.09, EtOH)); IR (microscope) 3070, 2957, 2933, 2912, 2855, 1771, 1426, 1361, 1346, 1174, 1110 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.68-7.60 (m, 4H, Ar-$\underline{H}$), 7.47-7.33 (m, 6H, Ar-$\underline{H}$), 4.63-4.54 (m, 1H, H-5), 3.86 (dd, 1H, *J* = 11.3, 3.3 Hz, 1 X H-6), 3.67 (dd, 1H, *J* = 11.3, 3.3 Hz, 1 X H-6), 2.65 (ddd, 1H, *J* = 17.4, 9.9, 7.2 Hz, 1 X H-3), 2.49 (ddd, 1H, *J* = 17.4, 9.9, 7.2 Hz, 1 X H-3), 2.34-2.13 (m, 2H, H-4), 1.05 (s, 9H, 1′ $(\mathrm{C\underline{H}_3})_3$); ^{13}C NMR ($\mathrm{CDCl_3}$, 125 MHz) δ 177.5 (C-2), 135.6 ($\mathrm{\underline{C}H}$), 135.5 ($\mathrm{\underline{C}H}$) 133.0 (*ipso*-$\mathrm{\underline{C}}$), 132.4 (*ipso*-$\mathrm{\underline{C}}$), 129.9 ($\mathrm{\underline{C}H}$), 127.8 ($\mathrm{\underline{C}H}$), 79.9 (C-5), 65.5 (C-6), 28.6 ($\mathrm{\underline{C}H_2}$), 26.8 ($\mathrm{C(\underline{C}H_3)_3}$), 23.7 ($\mathrm{\underline{C}H_2}$), 19.2 ($\mathrm{\underline{C}(CH_3)_3}$); HRMS (ES) calcd for $\mathrm{C_{21}H_{26}O_3Si}$ 354.1651, found 377.1542 $[\mathrm{M+Na}]^+$.

(+)-(3*S*,5*S*)-[(2*E*,4*E*)-Hexa-2,4-dien-1-yl]-5-[(*tert*-Butyl-diphenylsilanyloxy)methyl]dihydrofuran-2(*3H*)-one (119)



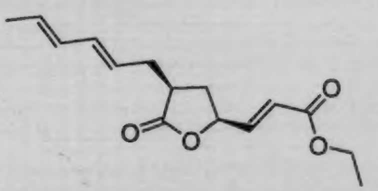
The known compound **119**[159] was prepared using the method described therein. A cooled solution (-78 °C) of NaHMDS (1.0 M in THF, 3.74 mL, 3.74 mmol) was added dropwise to a cooled (-78 °C) solution of **118** (1.288 g, 3.63 mmol) in dry THF (15 mL) over 15 min. The reaction mixture was allowed to stir for 15 min before a cooled (-78 °C) crude solution of 2*E*,4*E*-hexadienyl bromide (585 mg, 3.63 mmol) in dry THF (4 mL) was added quickly and the solution was left to stir for 30 min. The resulting mixture was added dropwise *via* a cannula to cooled (-78 °C) stirred solution of LiHMDS (1.0 M in THF, 3.88 mL, 3.88 mmol) and left to stir for 30 min before a cooled (-78 °C) solution of 2-bromo-2-methylpropane (418 μL, 3.63 mmol) in dry THF (4 mL) was added and the solution was left to stir for 40 min. The reaction was quenched by the addition of sat'd ammonium chloride (10 mL) and the reaction mixture was allowed to warm to rt. $\mathrm{H_2O}$ (10 mL) Was added to dissolve the solids, this mixture was then poured into $\mathrm{Et_2O}$ (15 mL) and the layers separated. The organic layer was washed with sat'd ammonium chloride (10 mL) and brine (10 mL) the aqueous layers were combined and back extracted with $\mathrm{Et_2O}$ (25 mL). The organic fractions were combined and dried ($MgSO_4$), filtered and the solvent evaporated under reduced pressure to give 1.7997 g of crude product, which by ^{1}H-NMR contained a mixture of the desired *trans* product, as well as the undesired *cis* (R_f 0.21 on SiO_2, 1:9/EtOAc:hexanes) and dialkylated (R_f 0.33 on SiO_2, 1:9/EtOAc:hexanes) products. The product was purified by flash chromatography (SiO_2, hexanes after some time switching to 5% EtOAc in hexanes) to yield **119** (515 mg, 32%) as a clear colourless oil. (R_f 0.13 on SiO_2, 1:9/EtOAc:hexanes). $[\alpha]_D^{20}$ +41.39° (*c* 1.20, $CHCl_3$) (lit.[73] $[\alpha]_D^{20}$ +42.9° (*c* 0.69, $CHCl_3$)); IR (microscope) 3071, 3049, 3017, 2957, 2931, 2857, 1774, 1589, 1472, 1114 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.69-7.64 (m, 4H, Ar-$\underline{H}$), 7.46-7.36 (m, 6H, Ar-$\underline{H}$), 6.09-5.93 (m, 2H, H-3′ & H-4′), 5.61 (dq, 1H, *J* = 13.2, 6.6 Hz, H-5′), 5.46 (dt, 1H, *J* = 14.4, 7.2 Hz, H-2′), 4.45 (dddd, 1H, *J* = 10.0, 6.4, 3.6, 3.6 Hz, H-5), 3.85 (dd, 1H, *J* = 11.6, 3.6 Hz, 1 X H-6), 3.70 (dd, 1H, *J* = 11.6, 4.0 Hz, 1 X H-6), 2.71 (dddd, 1H, *J* = 15.6, 11.6, 8.8, 4.4 Hz, H-3), 2.61-2.56 (m, 1H, 1 X H-1′), 2.33-2.20 (m, 2H, 1 X H1′ & 1 X H-4), 1.97-1.86 (m, 1H, 1 X H-4), 1.72 (d, 3H, *J* = 6.6 Hz, H-6′), 1.05 (s, 9H, $(C\underline{H}_3)_3$); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 178.0 (C-2), 135.7 ($\underline{C}$H), 135.6 ($\underline{C}$H), 133.2 ($\underline{C}$H), 133.1 (*ipso*-$\underline{C}$), 132.8 (*ispo*-$\underline{C}$), 131.1 ($\underline{C}$H), 129.8 ($\underline{C}$H), 128.5 ($\underline{C}$H), 127.8 ($\underline{C}$H), 126.8 ($\underline{C}$H), 78.5 (C-5), 64.6 (C-6), 40.6 (C-3), 33.3 ($\underline{C}H_2$), 29.3 ($\underline{C}H_2$), 26.7 ($C(\underline{C}H_3)_3$), 19.3 ($\underline{C}(CH_3)_3$), 18.0 (C-6′); HRMS (ES) calcd for $C_{27}H_{34}O_3Si$ 434.2277, found 457.2166 $[M+Na]^+$.

(+)-(3*S*,5*S*)-3-[(2*E*,4*E*)-Hexa-2,4-dien-1-yl]-5-(hydroxymethyl)dihydrofuran-2(3*H*)-one (120)



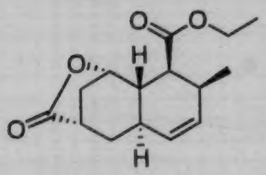
The known compound **120**[159] was prepared using the method described therein. TBAF (1.0 M in THF, 51 mL, 51 mmol) was added dropwise to a cooled (0 °C) stirred solution of **119** (20.1404 g, 46.34 mmol) in dry THF (200 mL) and the reaction mixture was warmed to rt over 3 h before diluting with Et_2O (200 mL). The mixture was washed with a 3 M citric acid sol'n (3 X 80 mL). The aqueous layers were combined and back extracted with Et_2O (4 X 80 mL). The organic fractions were combined, dried ($MgSO_4$), filtered and the solvent evaporated under reduced pressure. The crude mixture was purified by flash chromatography (SiO_2, 1:1/Et_2O:pentane to load and switching to 95% Et_2O in pentane) to yield **120** (7.6282 g, 84%) (R_f 0.25 on SiO_2, 5% pentane in Et_2O). $[\alpha]^{20}_{D}$ +62.49° (*c* 1.38, MeOH) (lit.[159] $[\alpha]^{20}_{D}$ +43° (*c* 0.04, MeOH)); IR (microscope) 2277 (br), 3020, 2933, 1758, 1452, 1436, 1184 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 6.15-5.85 (m, 2H, H-3′ & H-4′), 5.63 (dq, 1H, *J* = 13.5, 6.5 Hz, H-5′), 5.90 (dt, 1H, *J* = 14.7, 7.8 Hz, H-2′), 4.45 (dddd, 1H, *J* = 10.2, 6.3, 5.1, 3.0 Hz, H-5), 3.88 (dd, 1H, *J* = 12.6, 3.0 Hz, 1 X H-6), 3.60 (dd, 1H, *J* = 12.6, 3.6 Hz, 1 X H-6), 2.74 (dddd, 1H, *J* = 15.9, 11.4, 9.0, 4.2 Hz, H-3), 2.68-2.55 (m, 1H, 1 X H-1′), 2.33-2.20 (m, 2H, 1 X H-1′ & 1 X H-4), 1.88-1.75 (m, 1H, 1 X H-4), 1.72 (d, 3H, *J* = 6.5 Hz, H-6′); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 178.4 (C-2), 133.2 ($\underline{C}H$), 131.0 ($\underline{C}H$), 128.6 ($\underline{C}H$), 126.6 ($\underline{C}H$), 79.2 (C-5), 63.7 (C-6), 40.7 (C-3), 33.2 ($\underline{C}H_2$), 29.1 ($\underline{C}H_2$), 18.0 (C-6′); HRMS (EI) calcd for $C_{11}H_{16}O_3$ 196.1099, found 196.1097 $[M]^+$.

Ethyl (2*E*)-3-{(2*S*,4*S*)-4-[(2*E*,4*E*)-hexa-2,4-dien-1-yl]-5-oxotetrahydrofuran-2-yl}acrylate (121)



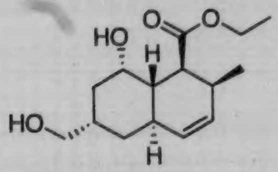
The known compound **121**[159] was prepared using the method described therein. DMSO (9.10 mL, 128.3 mmol) was added to a cooled (-78 °C) solution of oxalyl chloride (4.74 mL, 54.4 mmol) in dry CH_2Cl_2 (56.5 mL) over 25 min After 20 min a solution of the alcohol **120** (7.6282 g, 38.87 mmol) in dry THF (100 mL) was added over 1 h and left to stir for another 20 min A solution of diisopropylethylamine (27 mL, 155.5 mmol) was added over 15 min and allowed to stir for 10 min before warming to -5 °C when the mixture was added *via* a cannula to a cooled (0 °C), stirred solution of (carbethoxymethylene)triphenylphosphorane (33.37 g, 77 mmol) in dry THF (120 mL) over 1 h and allowed to stir and warm to rt for 20 h excluding light. The solvent was evaporated under reduced pressure and the remaining mixture dissolved in EtOAc, and washed twice with 2N HCl. The aqueous fractions were back extracted with EtOAc and the organic fractions combined, washed with brine, dried ($MgSO_4$), filtered and the solvents removed under reduced pressure. The mixture was purified by flash chromatography (SiO_2, 1:9/EtOAc:hexanes then switching to 1:3/EtOAc:hexanes) to yield pure **121** (R_f 0.35 on SiO_2, 1:3/EtOAc:hexanes) after 3 columns. By GC-MS **121** was the major peak with (t_r 20.24 min); $[\alpha]_D^{20}$ +61.40° (c 1.46, $CHCl_3$) (lit.[73] $[\alpha]_D^{20}$ +107.0° (c 6.24, $CHCl_3$)); IR (microscope) 2982, 2935, 1777, 1720, 1664, 1447, 1304, 1269, 1172 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.87 (dd, 1H, J = 15.6, 4.8 Hz, C$\underline{H}$=$CHCO_2Et$), 6.09 (dd, 1H, J = 15.6 Hz, 1.6 Hz, CH=C$\underline{H}CO_2Et$), 6.09-5.93 (m, 2H, H-3′ & H-4′), 5.63 (dq, 1H, J = 13.6, 6.4 Hz, H-5′), 5.90 (dt, 1H, J = 14.0, 7.2 Hz, H-2′), 4.93 (ddddd, 1H, *J* = 12.0, 10.4, 8.0, 5.2, 1.6 Hz, H-2), 4.21 (q, 2H, *J* = 7.2 Hz, $OC\underline{H}_2CH_3$), 2.70 (dddd, 1H, *J* = 16.0, 11.6, 8.8, 4.4 Hz, H-4), 2.65-2.57 (m, 1H, 1 X H-1′), 2.57-2.49 (m, 1H, 1 X H-1′), 2.31-2.21 (m, 1H, 1 X H-3), 1.78-1.67 (m, 1H, 1 X H-3), 1.72 (d, 3H, *J* = 6.4 Hz, H-6′), 1.28 (t, 3H, *J* = 7.2 Hz, $OCH_2C\underline{H}_3$); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 177.1 (C-5), 165.6 ($\underline{C}O_2Et$), 143.4 ($\underline{C}H{=}CHCO_2Et$), 133.6 ($\underline{C}H$), 130.9 ($\underline{C}H$), 128.9 ($\underline{C}H$), 126.0 ($\underline{C}H$), 122.2 ($CH{=}\underline{C}HCO_2Et$), 76.1 (C-2), 60.7 ($O\underline{C}H_2CH_3$), 40.5 (C-4), 34.1 ($\underline{C}H_2$), 33.0 ($\underline{C}H_2$), 18.0 (C-6′), 14.2 ($OCH_2\underline{C}H_3$); HRMS (EI) calcd for $C_{15}H_{20}O_4$ 264.1362, found 264.1364 $[M]^+$.

Ethyl (1*S*,2*S*,4a*R*,6*S*,8*S*,8a*S*)-1,2,4a,5,6,7,8,8a-octahydro-2-methyl-6,8-naphthalenecarbolactone-1-carboxylate (122)



The known compound **122**[159] was prepared using the method described therein. A round bottom flask was washed with HMDS and dried overnight in the oven (120 °C). After cooling to rt, **121** (4.1261 g, 15.6 mmol), mesitylene (450 mL) and butylated hydroxytoluene (447 mg) were added and the mixture refluxed for 20 min under argon. A drying tube ($CaCl_2$) was added and the mixture heated for 11 days before cooling and decanting the solvent and removing it under reduced pressure. The mixture was partially purified by flash chromatography (SiO_2, 1:1:8/MeCN:CH_2Cl_2:toluene). This semi purified compound was further purified by flash chromatography (SiO_2, 3:7/Et_2O:pentane) and (SiO_2, 2:3/Et_2O:pentane) to yield pure **122** (3.1512 g, 76%) (R_f 0.52 on SiO_2, 1:1/Et_2O:pentane). By GC-MS **121** was the major peak with (t_r 20.18 min); $[\alpha]_D^{20}$ +231.83° (*c* 2.24, $CHCl_3$) (lit.[159] $[\alpha]_D^{20}$ +260° (*c* 1.03, $CHCl_3$)); IR (microscope) 3028, 2986, 2965, 2933, 2899, 2859, 1767, 1724 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 5.49-5.42 (m, 2H, H-3 & H-4), 5.00 (d, 1H, *J* = 6.0 Hz, H-8eq), 4.12 (q, 2H, *J* = 7.2 Hz, $OC\underline{H}_2CH_3$), 2.80 (dd, 1H *J* = 11.6, 6.8 Hz, H-1), 2.69-2.60 (m, 2H, H-6eq & H-2), 2.44-2.24 (m, 1H, H-7eq), 2.35-2.25 (m, 1H, H-4a), 2.03-1.95 (m, 1H, H-5eq), 1.83 (d, 1H, *J* = 12.0 Hz, H-7ax), 1.69 (dd, 1H, *J* = 11.6 Hz, H-8a), 1.36 (ddd, 1H, *J* = 13.2, 13.2, 2.0 Hz, H-5ax), 1.23 (t, 3H, *J* = 7.2 Hz, $OCH_2C\underline{H}_3$), 0.84 (d, 3H, *J* = 7.2 Hz, 2-$C\underline{H}_3$); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 178.7 (6-$\underline{C}$=O), 172.9 (1-$\underline{C}$=O), 131.2 ($\underline{C}$H=CH), 128.0 ($\underline{C}$H=CH), 77.8 (C-8), 60.3 ($O\underline{C}H_2CH_3$), 46.0 (C-1), 39.2 (C-8a), 39.1 (C-6), 38.7 (C-7), 35.0 (C-4a), 32.6 (C-2), 31.8 (C-5), 17.2 (2-$\underline{C}H_3$), 14.2 ($OCH_2\underline{C}H_3$); HRMS (EI) calcd for $C_{15}H_{20}O_4$ 264.1362, found 264.1361 $[M]^+$.

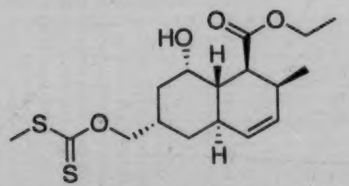
Ethyl (1*S*,2*S*,4a*R*,6*S*,8*S*,8a*S*)-8-hydroxy-6-(hydroxymethyl)-2-methyl-1,2,4a,5,6,7,8,8a-octahydronaphthalene-1-carboxylate (123)



The known compound **123**[73,159] was prepared using a modified version of the method of Witter.[73] $LiBEt_3H$ (1.0 M in THF, 17.67 mL, 17.67 mmol) was added slowly to a cooled (0 °C) stirred solution of **122** (4.2471g, 16.1 mmol) in dry THF (230 mL) over 10 min and left to stir for and additional 30 min. Then H_2O (4.6 mL), 2 N NaOH (8.9 mL) and 30% H_2O_2 (8.9 mL) were added and the reaction mixture was stirred for 1 h before diluting with Et_2O (230 mL) and washing with brine. The aqueous layer was back extracted with Et_2O (3 X 200 mL), the organic fractions were combined, dried ($MgSO_4$), filtered and the solvent evaporated under reduced pressure. By ^1H-NMR there appeared to be an aldehyde present from incomplete reduction to the primary alcohol, so the mixture was taken up in

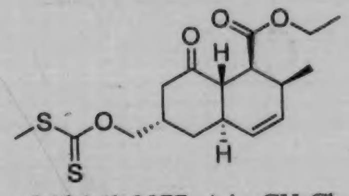
EtOAc (50 mL) and $NaBH_4$ (152 mg, 4.02 mmol) added and the reaction mixture stirred for 2 h before diluting with EtOAc (200 mL) and washing with 1 N HCl (150 mL). The aqueous fraction was back extracted with EtOAc (200 mL) and the organic fractions combined, dried ($MgSO_4$), filtered and the solvent removed under reduced pressure. The mixture was purified flash chromatography (SiO_2, loaded with 1:1/Et_2O:pentane and ran in 6% pentane in Et_2O) to yield **123** (2.20 g, 51%) (R_f 0.22 on SiO_2, 9:1/Et_2O:pentane). $[\alpha]_D^{20}$ +150.57° (*c* 1.10, $CHCl_3$) (lit.[73] $[\alpha]_D^{20}$ +142.0° (*c* 0.29, $CHCl_3$)); IR (microscope) 3245 (br), 3021, 3003, 2969, 2907, 2844, 1720 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ; 5.52 (ddd, 1H, *J* = 9.8, 4.5, 2.7 Hz, C$\underline{H}$=CH), 5.38 (d, 1H, *J* = 9.8 Hz, C$\underline{H}$=CH), 4.25 (ddd, 1H, *J* = 2.3 Hz, H-8eq), 4.20-4.05 (m, 2H, OC$\underline{H}_2$CH$_3$), 3.75 (d, 2H, *J* = 4.8 Hz, C$\underline{H}_2$OH), 2.81 (dd, 1H *J* = 11.7, 6.0 Hz, H-1), 2.73 (br s, 2H, 2 X O$\underline{H}$), 2.66-2.54 (m, 1H, H-2), 2.54-2.41 (m, 1H, H-4a), 2.01-1.92 (m, 1H, H-6), 1.91-1.85 (m, 2H, H-7), 1.84-1.75 (m, 1H, H-5eq), 1.48 (ddd, 1H, *J* = 11.1, 11.1, 2.3 Hz, H-8a), 1.34 (ddd, 1H, *J* = 13.5, 13.2, 5.7 Hz, H-5ax), 1.25 (t, 3H, *J* = 7.2 Hz, OCH$_2$C$\underline{H}_3$), 0.84 (d, 3H, *J* = 7.2 Hz, 2-C$\underline{H}_3$); ^{13}C NMR ($CDCl_3$, 100 MHz) δ; 173.9 (1-$\underline{C}$=O), 131.2 ($\underline{C}$H=CH), 130.1 ($\underline{C}$H=CH), 67.7 ($\underline{C}$H$_2$OH), 65.7 (C-8), 59.9 (O$\underline{C}$H$_2$CH$_3$), 45.0 (C-1), 39.7 (C-8a), 35.7 (C-7), 35.2 (C-5), 33.9 (C-6), 32.4 (C-2), 30.4 (C-4a), 17.6 (2-$\underline{C}$H$_3$), 14.2 (OCH$_2\underline{C}$H$_3$); HRMS (ES) calcd for $C_{15}H_{24}O_4$ 268.1675, found 291.1569 $[M+Na]^+$.

Ethyl(1*S*,2*S*,4a*R*,6*S*,8*S*,8a*S*)-8-hydroxy-2-methyl-6({[(methylthio)carbonothioyl]oxy}methyl)-1,2,4a,5,6,7,8,8a-octahydronaphthalene-1-carboxylate (124)



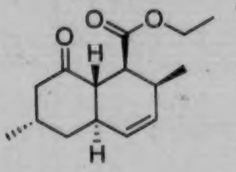
The known compound **124**[73] was prepared using a modified version of the method of Di Cesare and Gross.[160] A solution of 4 N NaOH (66 mL, 264 mmol) was quickly added to a solution of the diol **123** (2.2 g, 8.2 mmol) and tetrabutylammonium hydrogen sulfate (3.09 g, 9 mmol) in dry benzene (66 mL). Then CS_2 (1 mL, 16.57 mmol) and MeI (0.76 mL, 12.2 mmol) were quickly added to the solution, which was left to stir for 10 min before ice (132 g) and Et_2O (132 mL) were added and the reaction mixture stirred for an additional 5 min The mixture was then diluted with Et_2O (200 mL) and the layers separated. The aqueous layer was back extracted with Et_2O (200 mL) and the organic fractions combined, washed with brine, dried ($MgSO_4$), filtered and the solvent evaporated under reduced pressure to yield 3.3077 g of crude product. By ^{1}H-NMR the crude material looked greater than 90% pure and was used in the next reaction without further purification. $[\alpha]_D^{20}$ +164.39° (*c* 2.18, $CHCl_3$) (lit.[73] $[\alpha]_D^{20}$ +162.9° (*c* 0.42, $CHCl_3$)); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 5.55 (ddd, 1H, *J* = 9.8, 4.5, 2.7 Hz, C$\underline{H}$=CH), 5.36 (d, 1H, *J* = 9.8 Hz, C$\underline{H}$=CH), 4.88 (dd, 1H, *J* = 10.8, 9.0 Hz, 1 X C$\underline{H}_2$OC(S)S), 4.74 (dd, 1H, *J* = 10.8, 6.0 Hz, 1 X C$\underline{H}_2$OC(S)S), 4.32-4.26 (m, 1H, H-8eq), 4.20-4.04 (m, 2H, OC$\underline{H}_2$CH$_3$), 2.83 (dd, 1H *J* = 11.7, 6.0 Hz, H-1), 2.66-2.54 (m, 1H, H-2), 2.54 (s, 3H, SC$\underline{H}_3$), 2.54-2.24 (m, 2H, H-4a & H-6), 1.92-1.85 (m, 3H, H-7 & H-5eq), 1.60 (br s, 1H, O$\underline{H}$), 1.42 (ddd, 1H, *J* = 11.4, 11.4, 2.1 Hz, H-8a), 1.30 (ddd, 1H, *J* = 13.5, 13.5, 5.1 Hz, H-5ax), 1.24 (t, 3H, *J* = 7.2 Hz, OCH$_2$C$\underline{H}_3$), 0.84 (d, 3H, *J* = 7.2 Hz, 2-C$\underline{H}_3$).

Ethyl(1*S*,2*S*,4a*R*,6*S*,8a*S*)-2-methyl-6-({[(methylthio)carbonothioyl]oxy}methyl)-8-oxo-1,2,4a,5,6,7,8,8a-octahydronaphthalene-1-carboxylate (125)



The known compound **125**[73] was prepared using a modified version of the method of Furber and Mander.[161] PDC (4.626 g, 12.3 mmol) was added to a crude solution of **124** (3.3077 g) in CH_2Cl_2 (66 mL) and the reaction mixture left to stir for 44 h before evaporating the solvent under reduced pressure and filtering the material through a plug (SiO_2, 1:1/Et_2O:pentane) to remove the PDC to give a yellow oil. This was purified by flash chromatography (SiO_2, 1:9/Et_2O:pentane to load and run, after some time switching to 3:7/Et_2O pentane) to yield **125** (2.4677 g, 84%) as a white waxy solid (SiO_2, 3:7/Et_2O:pentane, R_f 0.24). IR (microscope) 3021, 2986, 2972, 2957, 2930, 2901, 2828, 1736, 1713 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 5.55 (ddd, 1H, J = 9.8, 4.5, 2.7 Hz, $C\underline{H}$=CH), 5.42 (ddd, 1H, J = 9.8, 1.8, 1.5 Hz, $C\underline{H}$=CH), 4.51 (dd, 1H, J = 11.1, 7.8 Hz, 1 X $C\underline{H}_2OC(S)S$), 4.30 (dd, 1H, J = 11.1, 6.9 Hz, 1 X $C\underline{H}_2OC(S)S$), 4.22-4.04 (m, 2H, $OC\underline{H}_2CH_3$), 2.90-2.56 (m, 5H, H-1 & H-2 & H-4a & 1 X H-7 & H-8a), 2.52 (s, 3H, $SC\underline{H}_3$), 2.35 (ddd, 1H, J = 13.5, 2.1, 1.8 Hz, 1 X H-7), 2.32-2.20 (m, 1H, H-6), 2.00-1.90 (m, 1H, H-5eq), 1.77 (ddd, 1H, J = 13.2, 13.2, 5.4 Hz, H-5ax), 1.24 (t, 3H, J = 7.2 Hz, $OCH_2C\underline{H}_3$), 0.87 (d, 3H, J = 7.2 Hz, 2-$C\underline{H}_3$); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 215.7 ($S\underline{C}(S)O$), 208.9 (C-8), 173.4 ($\underline{C}$=O), 132.4 ($\underline{C}$H=CH), 128.0 ($\underline{C}$H=CH), 74.4 ($\underline{C}H_2OC(S)S$), 60.2 ($O\underline{C}H_2CH_3$), 49.2 ($\underline{C}$H), 42.9 (C-7), 42.6 ($\underline{C}$H), 38.0 (C-6), 35.4 ($\underline{C}$H), 32.9 (C-5), 31.1 ($\underline{C}$H), 19.1 ($S\underline{C}H_3$), 17.7 (2-$\underline{C}H_3$), 14.2 ($OCH_2\underline{C}H_3$); HRMS (EI) calcd for $C_{17}H_{24}O_4S_2$ 356.1116, found 356.1111 $[M]^+$.

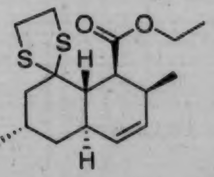
Ethyl (1*S*,2*S*,4a*R*,6*S*,8a*S*)-2,6-dimethyl-8-oxo-1,2,4a,5,6,7,8,8a-octahydronaphthalene-1-carboxylate (126)



The known compound **126**[73] was prepared using the method of Barton *et al.*[162] **125** (53.1 mg, 149 μmol) Was dissolved in dry *p*-cymene (5 mL) in a 3-neck flask fitted with a condenser and argon was bubbled through the solution for 25 min before the mixture was heated to 150 °C. To this mixture was slowly added a solution of Bu_3SnH (0.321 mL, 1.19 mmol) in dry *p*-cymene (4.7 mL) (this had argon bubbling through it for 15 min prior to addition) over 1 h. The mixture was allowed to stir at 150 °C overnight. The mixture was cooled and transferred to a round bottom flask and the solvent evaporated under reduced pressure. The crude mixture was purified by flash chromatography (SiO_2, 1:9/Et_2O:pentane to load then switching to 1:4/Et_2O:pentane) to yield **126** (27.5 mg, 74%) (SiO_2, 3:7/Et_2O:pentane, R_f 0.36). Using standard GC-MS method **126** was a single peak with (t_r 18.28 min); $[\alpha]_D^{20}$ +209.41° (*c* 0.73, $CHCl_3$) (lit.[73] $[\alpha]_D^{20}$ +80.8° (*c* 0.13, $CHCl_3$)); ; IR (microscope) 3016, 2969, 2949, 2918, 2851, 2837, 1731, 1706, 1448 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 5.61 (ddd, 1H, *J* = 10.0, 4.8, 2.8 Hz, C$\underline{H}$=CH), 5.41 (ddd, 1H, *J* = 10.0, 2.0, 1.6 Hz, C$\underline{H}$=CH), 4.20-4.05 (m, 2H, OC$\underline{H_2}$CH$_3$), 2.79 (dd, 1H, *J* = 11.6, 6.8 Hz, H-1), 2.71 (dd, 1H, *J* = 12.8, 6.8 Hz, H-8a), 2.65-2.48 (m, 3H, H-2 & H-7), 2.34-2.24 (m, 1H H-6), 2.12 (ddd, 1H, *J* = 12.8, 2.0, 2.0 Hz, H-4a), 1.75 (ddd, 1H, *J* = 13.2, 13.2, 4.8 Hz, H-5ax), 1.69-1.62 (m, 1H, H-5eq), 1.23 (t, 3H, *J* = 7.2 Hz, OCH$_2$C$\underline{H_3}$), 0.94 (d, 3H, *J* = 7.2 Hz, 6-C$\underline{H_3}$), 0.85 (d, 3H, *J* = 7.2 Hz, 2-C$\underline{H_3}$); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 210.4 (C-8), 173.7 ($\underline{C}$=O), 132.1 ($\underline{C}$H=CH), 128.8 ($\underline{C}$H=CH), 60.1 (O$\underline{C}$H$_2$CH$_3$), 49.5 ($\underline{C}$H), 48.0 ($\underline{C}$H$_2$), 42.8

($\underline{C}$H), 37.9 ($\underline{C}H_2$), 37.8 ($\underline{C}$H), 31.3 ($\underline{C}$H), 31.2 ($\underline{C}$H), 19.3 ($\underline{C}H_3$), 17.7 ($\underline{C}H_3$), 14.2 ($OCH_2\underline{C}H_3$); HRMS (EI) calcd for $C_{15}H_{22}O_3$ 250.1569, found 250.1561[M]$^+$.

Ethyl (1*S*, 2*S*, 4a*R*, 6*S*, 8a*S*)-1,2,4a, 5,6,7,8,8a-octahydro-8-(dimethylenedithio)-2,6-dimethylnaphthalen-1-carboxylate (127)



The known compound **127**[73] was prepared using a modified version of the method of Falck and coworkers.[163] Freshly distilled 1,2-ethanedithiol (263 µL, 3.12 mmol) was added to a cooled (0 °C) solution of **126** (390.6 mg, 1.56 mmol) in dry CH_2Cl_2 (14 mL) followed by the addition of freshly distilled BF_3•OEt_2 (198 µL, 1.56 mmol). The reaction mixture was allowed to warm to rt overnight before it was diluted with CH_2Cl_2 and washed sequentially with 1 N NaOH, 1 N HCl, H_2O and brine. The organic solvent was evaporated under reduced pressure and the mixture purified by flash chromatography (SiO_2, 5% Et_2O in pentane) to yield **127** (389.5 mg, 76%) (SiO_2 10% Et_2O in pentane, R_f 0.30). $[\alpha]_D^{20}$ +68.56° (*c* 1.73, $CHCl_3$) (lit.[73] $[\alpha]_D^{20}$ +84.3° (*c* 0.22, $CHCl_3$)); IR (microscope) 3026, 2958, 2925, 2876, 2847, 1728, 1637, 1457 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 5.60 (s, 2H, C$\underline{H}$=C$\underline{H}$), 4.18-4.00 (m, 2H, $OC\underline{H}_2CH_3$), 3.42-3.17 (m, 4H, $SC\underline{H}_2C\underline{H}_2S$), 3.08 (dd, 1H, *J* = 7.8, 6.3 Hz, H-1), 2.45 (dq, 1H, *J* = 7.2, 6.9 Hz, H-2), 2.30-2.21 (m, 2H, H-7), 2.18 (dd, 1H, *J* = 10.8, 6.3 Hz, H-8a), 2.16-1.98 (m, 2H, H-4a & H-6), 1.77-1.69 (m, 1H, H-5eq), 1.62 (ddd, 1H, *J* = 12.6, 12.6, 5.4 Hz, H-5ax), 1.22 (t, 3H, *J* = 7.2 Hz, $OCH_2C\underline{H}_3$), 1.19 (d, 3H, *J* = 7.5 Hz, 6-$C\underline{H}_3$), 1.08 (d, 3H, 7.2 Hz, 2-$C\underline{H}_3$); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 176.0 ($\underline{C}$=O), 134.1 ($\underline{C}$H=CH), 133.3 ($\underline{C}$H=CH), 72.7, (C-8), 59.9 ($O\underline{C}H_2CH_3$), 54.2 ($\underline{C}$H), 50.9 ($\underline{C}H_2$), 49.7 ($\underline{C}$H), 40.9 ($\underline{C}H_2$), 38.5 ($\underline{C}H_2$), 38.0 ($\underline{C}H_2$), 33.7 ($\underline{C}$H), 31.9 ($\underline{C}$H), 29.5 ($\underline{C}$H), 20.4

($\underline{C}H_3$), 17.6 ($\underline{C}H_3$), 14.3 ($OCH_2\underline{C}H_3$); HRMS (EI) calcd for $C_{17}H_{26}O_2S_2$ 326.1374, found 326.1370 $[M]^+$.

Secondary deuterium kinetic isotope effects of Diels-Alder cyclisation of 74 and 78

Using the same methods as for the formation of the cyclised NAC-esters and for the transesterification to the ethyl esters, a 1:1 mixture of **74**:**78** (5.04 mg, 16 μmol) was combined in (300 μL MeOH and 700 μL pellet buffer, pH 7.8) and underwent the cyclisation, shaking (50 rpm) for 2 days at rt followed by 2 days at 50 °C. They were quenched to give 7.5 mg of crude product, which consisted of a mixture of 3 products by ^{1}H-NMR as well as the unreacted triene. This mixture in CH_2Cl_2 (2 mL) was transesterified with NaOEt (0.15 mL of a 0.5M sol'n) over 24 h before working up (^{1}H-NMR showed all 3 products and transesterified triene ethyl ester **112**) and purifying by preparative TLC (SiO_2, 5% Et_2O in pentane). By analyzing the mixture by GC-MS and confirming each peaks fragmentation pattern using a scanning MS which gave a weak M^+, the sample was run monitoring only the M^+ (236 and 237 *m/z*). Each peak contains the mass 236 and 237. To determine the amount of the 237 mass that is contributed by the deuterated species, 16.7% of the area of the 237 contribution was removed to remove the ^{13}C contribution from the 236 mass. The ratio of the 236 peak divided by the calculated deuterated 237 peak (each done in triplicate) and this number was divided by the ratio of proonated/deuterated starting materials which gave an inverse kinetic isotope effect for the 3 products (**114**, **113**, **79**) of 0.906 ± 0.009, 0.934 ± 0.005 and 0.933 ± 0.023 (see appendix B) respectively indicating a concerted Diels-Alder cyclisation.

3.3 FERMENTATION AND ENZYMATIC REACTIONS

Fermentation of *Aspergillus nidulans lovB, lovB+C and lovC* strains

Aspergillus nidulans lovB, *lovB+C* and *lovC* strains were provided by Professor C. R. Hutchinson (University of Wisconsin, now with Kosan Biosciences). The *lovB* strain (UAMH 8965 or WMH1738) had been transformed with a plasmid containing the LNKS gene from *A. terreus* controlled by the cyclopentanone-inducible promoter *alcA* as well as the uridine marker necessary for the biosynthesis of uridine which is lacking in the host strain. The *lovB+C* strain (UAMH 9442 or WMH1750) refers to the *lovB* strain with the addition of a plasmid containing the *lovC* gene controlled by the *alcA* promoter as well as the phleomycin resistance gene. The *lovC* strain contains a plasmid containing the *lovC* gene from *A. terreus* controlled by the *alcA* promoter. For the *lovC* strain it is necessary to add 2.4 g/L uridine and 2.2 g/L uracil for growth and expression. All solutions, tools, and glassware were autoclaved prior to use. Spores of the above strains were obtained either from slants previously prepared by Dr. Karine Auclair or *via* inoculation of potato dextrose agar (PDA) plates (39 g/L PDA in Milli-Q water, autoclaved) and streptomycin (100 mg/L syringe filtered) with the appropriate spores which were grown until the colonies turned black (about 2 weeks). 10 mL Milli-Q H_2O was then added and the colonies were then rubbed with a glass rod to dislodge the spores. The spores were filtered through cheese cloth into a centrifuge tube and centrifuged at 2500 rpm and 4 °C for 20 min. The supernatant was removed and the spores resuspended in 1 mL Milli-Q water. The spore suspension was partitioned into 10 eppendorf tubes and stored at -78 °C. The spore suspension (10-20 μL) or part of the agar slant were inoculated into the growth media (20 g glucose, 20 g yeast extract, 1 g bactopeptone, 1 mL PABA solution (100 mg PABA in 100 mL Milli-Q water), diluted to 1 L with Milli-Q H_2O in a 2 L Erlenmeyer) and fermented at 30 °C, 200 rpm for 3 days before filtering the mycelia through Miracloth (Calbiochem, La Jolla, CA). The mycelia were washed with lactose solution (2 L of a 10% lactose solution in Milli-Q water) and transferred to the production media [1 mL trace elements solution (1 g $FeSO_4 \bullet 7H_2O$, 8.8 g $ZnSO_4 \bullet 7H_2O$, 400 mg $CuSO_4 \bullet 5H_2O$, 150 mg $MnSO_4 \bullet 4H_2O$, 100 mg $Na_2B_4O_7 \bullet 10\ H_2O$ and 50 mg $(NH_4)_6Mo_7O_{24} \bullet 4H_2O$, diluted to 1 L with Milli-Q water); 100 mL 10 X AMM salts solution (60 g $NaNO_3$, 5.2 g KCl, 15.2 g KH_2PO_4, diluted to 1 L pH adjusted to 6.5); 1 mL PABA solution (100 mg PABA in 100 mL Milli-Q water); 0.9 mL cyclopentanone (for induction); all of this was diluted to 875 mL total volume with Milli-Q water, and autoclaved before adding 2.5 mL $MgSO_4 \bullet 7H_2O$ (20% solution) and 25 mL sterile 40% lactose solution] and incubated at 30 °C, 200 rpm for 3-4 days for enzyme isolation experiments and 7 days for dihydromonacolin L (1) production (with feeding of 1 g NaOAc per day after the second day of production).

Preparation of cell free extracts of LovB and LovB+C (Method A)

A modified version of the method of Reeves and coworkers was used.[53] The mycelia from either the *lovB* or *lovB+C* strains were gravity filtered through 4 layers of cheese cloth and washed with 2 L Milli-Q water. The moist mycelia were frozen to -78 °C and freeze-dried overnight. At 4 °C, the proteins were extracted from the dried mycelia by grinding the mycelia in a mortar and pestle for 10-20 min with 40 mL extraction buffer (3.15 g Tris-HCl (dissolved in 300 mL Milli-Q water, pH adjusted to

8.0 with 1 M NaOH), 100 mL glycerol, 58.5 g NaCl, 1.0 g sodium ascorbate, 290 mg disodium EDTA, 155 mg DTT, 35 mg phenylmethylsulfonyl fluoride, 2.2 g polyvinylpolypyrolidone, 4 mg leupeptin, 17.5 mg chymostatin, 2.0 mg pepstatin A, 42 mg trypsin inhibitor, all diluted to a final volume of 1 L with Milli-Q H_2O giving a final pH of 6.8). The ground mycelia were then diluted with 10 mL extraction buffer and centrifuged (8000 rpm, 4 °C, 30 min) before filtering the supernatant through 4 layers of cheese cloth to afford the CFE. This produced active enzymes using the UV enzyme assay.

Preparation of cell free extracts of LovB and LovB+C (Method B)

The same procedure as for Method A was used except a 5 mM phosphate buffer (pH 6.6) with DTT (870 mg/L buffer) was used to extract the freeze-dried mycelia in place of the extraction buffer. This produced active enzymes using the UV enzyme assay.

Purification of LovB (LNKS)

A modified version of the method of Reeves and coworkers was used.[53] The LovB CFE was dialyzed with an amicon 200 (YM100, 100,000 MWCO) until the volume was ~20 mL. The concentrate was then loaded onto a Sephadex G-150 or G-200 (600-1000 mL) packed in column buffer (12.6 g Tris-HCl, 23.4 g NaCl, 740 mg sodium ascorbate, 1.5 g disodium EDTA, 4.0 mL Tween 80, 400 mL glycerol and 620 mg DTT, all diluted to 4 L with Milli-Q water) at 4 °C. The column was run at 0.5-1.0 mL/min collecting 8 mL fractions and monitoring at 280 nm. The pooled fractions were precipitated by slowly adding ammonium sulfate until a 60% saturation was reached. The off-white precipitate was centrifuged at 9000 rpm, 4 °C, 30 min twice. The supernatant was carefully removed by Pasteur pipette and the resulting pellet resuspended in a minimal amount of pellet buffer (610 mg Tris-HCl (dissolved in 125 mL Milli-Q water, pH adjusted to 8.0 with 1 M NaOH) 50 mg sodium ascorbate, 38 mg DTT, all diluted to a final volume of 250 mL with Milli-Q H_2O giving a final pH of 7.8) to yield the pure protein.

LovB UV enzyme assay

The blank was prepared by mixing 20 µL of a cofactor solution (1 mg SAM, 1 mg FAD, 1 mg NADPH dissolved in 1 mL Milli-Q water) with 500 µL of an enzyme solution with 300 µL Milli-Q water. The enzyme assay was prepared by mixing 20 µL of the cofactor solution with 500 µL of an enzyme solution, 50 µL of an Ac-CoA solution (1 mg/mL in Milli-Q water) and 250 µL of a malonyl-CoA solution (5 mg/mL in Milli-Q water). The enzyme activity was monitored by UV (365 nm) over a period of 20 h looking for an increase in absorbance indicating production of the pigments (**3** & **4**). The activity of LovB+C could also be monitored in the same way as the LovB as this organism also produces the pigments. (See appendix A for traces)

Studies on the cell free production of dihydromonacolin L (1) by *A. nidulans lovB+C*.

Trial 1

The CFE was prepared as described above (Method A). To the enzyme solution (80 mL) was added FAD (5 mg), SAM (1.5 mg), NADPH (33 mg), Ac-CoA (2.7 mg), malonyl-CoA (23 mg) and 1,2-^{14}C malonyl-CoA (0.45 µCi, 999,000 DPM). The mixture was placed on an orbital shaker (50 rpm) at rt for 17 h before it was acidified with 4 M HCl to pH 2. To this mixture was added **1** (17.3 mg) and the mixture extracted with CH_2Cl_2 (thrice). The organic fraction was dried (Na_2SO_4), filtered and evaporated under reduced pressure to yield the crude extract (21.8 mg). Part of the crude extract was purified by preparative TLC (EtOAc only). The spot corresponding to **1** was not radioactive.

Trial 2 (product extraction differences)

In a similar method to attempt 1, the CFE was prepared as described above (Method A). To the enzyme solution (50 mL) was added FAD (12.5 mg), SAM (12.5 mg), NADPH (12.5 mg), Ac-CoA (5.0 mg), malonyl-CoA (20 mg) and 1,2-^{14}C malonyl-CoA (0.45 μCi, 999,000 DPM). The mixture was placed on an orbital shaker (50 rpm) at rt for 23 h before it was acidified with 4 M HCl to pH 2. The mixture extracted with EtOAc (3 X 40 mL). The organic fraction was dried (Na_2SO_4), filtered and the solvent evaporated under reduced pressure to yield the crude extract. **1** Could not be detected by TLC. The crude extract was dissolved in toluene (15 mL) and refluxed with a soxhlet containing CaH_2 for 1 h to lactonize the open form. The reaction mixture was cooled and the solvent evaporated under reduced pressure. **1** (7.3 mg) Was added to the mixture and it was purified by preparative TLC (EtOAc only). The spot corresponding to **1** was not radioactive.

Trial 3 (enzyme preparation differences)

In a very similar method to attempt 2, the enzyme solution was prepared by washing the mycelia after expression of the proteins with a 5 mM phosphate buffer (pH 6.6). The mycelia were then frozen in N_2 (liq.) and ground with a mortar and pestle into a fine powder. Half the powder was used as a control and the other half was mixed with extraction buffer (60 mL). To the enzyme mixtures was added FAD (5 mg), SAM (1.5 mg), NADPH (33 mg), Ac-CoA (2.7 mg), malonyl-CoA (23 mg) and 1,2-^{14}C malonyl-CoA (0.45 μCi, 999,000 DPM). The mixtures were placed on an orbital shaker (50 rpm) at rt for 17 h before they were acidified with 4 M HCl to pH 2. The mixtures were extracted with EtOAc (thrice). The organic fractions were dried (Na_2SO_4), filtered and the solvent evaporated under reduced pressure to yield the crude extract. **1** Could not be detected by TLC. The crude extracts were dissolved in toluene (15 mL) and refluxed with a soxhlet containing CaH_2 for 1 h to lactonize the open form. The reaction mixtures were cooled and the solvent evaporated under reduced pressure. The mixtures were purified by preparative TLC (EtOAc only) and HPLC as described for **1**. The spot/peak corresponding to **1** were not radioactive.

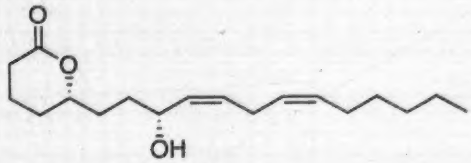
Trial 4 (centrifugation differences)

In a very similar method to attempt 2, the CFE was prepared as described above (Method A) with the exception that the crude extract was centrifuged at 1000 rpm for 30 min followed by 3000 rpm for 10 min before decanting to yield the CFE. To the enzyme mixture (20 mL) was added FAD (5 mg), SAM (1.4 mg), NADPH (33 mg), Ac-CoA (2.7 mg), malonyl-CoA (22.5 mg) and 1,2-^{14}C malonyl-CoA (0.45 μCi, 999,000 DPM). The mixture was placed on an orbital shaker (50 rpm) at rt for 17 h before it was acidified with 4 M HCl to pH 2. The mixture was extracted with EtOAc (3 X 40 mL). The organic fraction was dried (Na_2SO_4), filtered and the solvent evaporated under reduced pressure to yield the crude extract. **1** Could not be detected by TLC. The crude extract was dissolved in toluene (15 mL) and refluxed with a soxhlet containing CaH_2 for 1 h to lactonize the open form. The reaction mixture was cooled and the solvent evaporated under reduced pressure. To this mixture was added **1** (5.3 mg). The mixture was purified by preparative TLC (EtOAc only) followed by HPLC as described for **1**. The spot/peak corresponding to **1** were not radioactive.

Trial 5 (ATP differences)

In a very similar method to attempt 2, the CFE was prepared as described above (Method A). To the enzyme solution was added FAD (20 mg), SAM (6 mg), NADPH (40 mg), Ac-CoA (13 mg), malonyl-CoA (23 mg), 1,2-^{14}C malonyl-CoA (0.45 μCi, 999,000 DPM), and ATP (40 mg). The mixture was placed on an orbital shaker (50 rpm) at rt for 17 h before it was acidified with 4 M HCl to pH 2. The mixture was extracted with EtOAc (thrice). The organic fraction was dried (Na_2SO_4), filtered and evaporated under reduced pressure to yield the crude extract. Part of the crude extract was purified by HPLC as described for **1**. The peak corresponding to **1** was not radioactive.

Studies on cell free production of dihydromonacolin L (1) by *A. nidulans lovB* and *A. nidulans lovC*, isolation of psiAα 68



The CFE (as prepared by Method A) of each of the organisms were mixed for 30 min before FAD (5 mg), SAM (1.4 mg), NADPH (33 mg), Ac-CoA (2.7 mg) and malonyl-CoA (22.5 mg) were added. The mixture was placed on an orbital shaker (50 rpm) at rt for 40 h before it was acidified with 2 M HCl to pH 2. The mixture extracted with CH_2Cl_2 (thrice). The organic fraction was dried (Na_2SO_4), filtered and evaporated under reduced pressure to yield the crude extract. The crude extract was dissolved in toluene (15 mL) and refluxed with a soxhlet containing CaH_2 for 1 h to lactonize the open form. The reaction mixture was cooled and the solvent evaporated under reduced pressure. The mixture was purified by HPLC as described for **1**. A peak with the retention time and R_f (by TLC) corresponding to **1** was obtained. However, HRMS (EI) and (ES) led to a compound with the molecular formula $C_{18}H_{30}O_3$, one carbon less than **1**. ^{1}H-NMR spectra matched that of the reported data for psiAα as described by Nakanishi *et al.*[123] **1** Could not be detected.

In a similar attempt to the one just described except using the ultrafiltered CFE of LovB (YM100, 100,000 MWCO) and the ultrafiltered CFE of lovC (YM10, 10,000 MWCO). The psiAα was again isolated and **1** could not be detected.

Studies on cell free production of dihydromonacolin L (1) by incorporation of heptaketide-NAC 7 ester by *A. nidulans lovB* or *lovB+C*

Trial 1

The LovB CFE (25 mL) was prepared as described above (Method A). To the enzyme solution was added FAD (7 mg), NADPH (25 mg), malonyl-CoA (25 mg), 1,2-^{14}C malonyl-CoA (0.45 μCi, 999,000 DPM), and heptaketide-NAC ester (5 mg in 1 mL EtOH). The mixture was placed on an orbital shaker (50 rpm) at rt for 20 h before it was acidified with 4 M HCl to pH 2. The mixture extracted with CH_2Cl_2 (thrice). The organic fraction was dried (Na_2SO_4), filtered and evaporated under reduced pressure to yield the crude extract. Part of the crude extract was purified by HPLC as described for **1**. Heptaketide-NAC ester was recovered and no peak corresponding to **1** was detected.

Trial 2 (CFE preparation difference)

The LovB CFE was prepared as described above (Method B). To the enzyme solution was added heptaketide-NAC ester (5 mg in 1 mL EtOH) and the mixture incubated for 40 min at rt before FAD (7 mg), NADPH (25 mg), and 1,2-^{14}C malonyl-CoA (0.45 μCi, 999,000 DPM) were added. After 10 min malonyl-CoA (25 mg) was added. The mixture was placed on an orbital shaker (700 rpm) at rt for 20 h before it was acidified with 4 M HCl to pH 2. The mixture extracted with CH_2Cl_2 (thrice). The organic fraction was dried (Na_2SO_4), filtered and evaporated under reduced pressure to yield the crude extract. Part of the crude extract was purified by HPLC as described for **1**. Heptaketide-NAC ester and hydrolysed heptaketide **6** were recovered. However, **1** was not detected.

Trial 3 (LovB+C)

The LovB+C CFE (30 mL) was prepared as described above (Method A, with no DTT added to the extraction buffer). To the enzyme solution was added FAD (2 mg), NADPH (10 mg), Ac-CoA (5 mg) and half the heptaketide-NAC ester solution (5.1 mg in 400 μL EtOH, 900 μL H_2O, 1 drop 1 M HCl, 3 drops acetone) and the mixture incubated for 10 min at rt before half the solution of (1,2-^{14}C malonyl-CoA (0.90 μCi, 1,998,000 DPM) and malonyl-CoA (10 mg) in 1 mL H_2O) was added. The mixture was placed on an orbital shaker (50 rpm) at rt for 12 h. The other half of the malonyl-CoA solution was added. The reaction mixture was allowed to react for 21 h before it was acidified with 4 M HCl to pH 2. The mixture extracted with CH_2Cl_2 (thrice). The organic fraction was dried (Na_2SO_4), filtered and evaporated under reduced pressure to yield the crude extract. Part of the crude extract was purified by HPLC as described for **1**. Heptaketide-NAC ester was recovered. **1** Was not detected.

Studies on cell free production of dihydromonacolin L (1) by incorporation of heptaketide-CoA ester 12 by *A. nidulans lovB*

The LovB CFE (40 mL) was prepared as described above (Method A). To the enzyme solution was added FAD (1 mg) and NADPH (7.3 mg). To this mixture was added 100 μL of a heptaketide-CoA ester **12** solution (4.3 mg in 900 μL acidified H_2O H_2O and 100 μL EtOH) followed by 100 μL of the malonyl-CoA solution after 20 min (malonyl-CoA (7.5 mg) and 1,2-^{14}C malonyl-CoA (0.45 μCi, 999,000 DPM in 1 mL acidic water). The mixture was placed on an orbital shaker (50 rpm) at rt. The addition step was repeated each hour a total of 9 times before it was acidified with 4 M HCl to pH

2. The mixture extracted with CH_2Cl_2 (thrice). The organic fraction was dried (Na_2SO_4), filtered and evaporated under reduced pressure to yield the crude extract. Part of the crude extract was purified by HPLC as described for **1**, no peak corresponding to **1** was detected. To the other portion of the crude extract was added **1** (2 mg) and the mixture purified by HPLC as described for **1**. The dihydromonaclin L (**1**) collected was not radioactive.

Studies on cell free production of labeled dihydromonacolin L (1) by incorporation of ^{13}C labeled triketide-NAC ester 17 by *A. nidulans lovB+C*

The LovB+C CFE (25 mL) was prepared as described above (Method A). To the enzyme solution was added 5-^{13}C triketide-NAC ester (5 mg in 1 mL EtOH, prepared by Kris Rathwell[102]) and the mixture incubated for 1 h before FAD (50 mg), NADPH (77 mg), SAM (10 mg), malonyl-CoA (66 mg) were added. The mixture was placed on an orbital shaker (50 rpm) at rt for 20 h before it was acidified with 4 M HCl to pH 2. The mixture extracted with CH_2Cl_2 (thrice). The organic fraction was dried (Na_2SO_4), filtered and evaporated under reduced pressure to yield the crude extract. However, **1** was not detected.

Studies on cell free production of the labeled pigments 18a and 19a by incorporation of ^{13}C labeled triketide-NAC ester 17 by *A. nidulans lovB*

The LovB CFE (35 mL) was prepared as described above (Method A). To the enzyme solution was added 5-^{13}C triketide-NAC ester (5 mg in 500 μL EtOH, prepared by Kris Rathwell[102]) and the mixture incubated for 1.5 h before FAD (20 mg), NADPH

(77 mg), SAM (10 mg), malonyl-CoA (56 mg) were added. The mixture was placed on an orbital shaker (50 rpm) at rt for 8 h before it was acidified with 4 M HCl to pH 2. The saturated pyrones **18** and **19** (0.3 mg each) were added to the mixture and the mixture was extracted with EtOAc (thrice). The organic fraction was dried (Na_2SO_4), filtered and evaporated under reduced pressure to yield the crude extract. The crude extract was dissolved in 3 mL EtOAc and a tip of 10% Pd/C was added followed by placing under an atmospheric pressure of H_2 for 12 h. The mixture was filtered though a pad of celite washing with EtOAc. The solvent was evaporated under reduced pressure to yield the crude reaction mixture. A ^{13}C label could be detected in (**18** and **19**). It appears a ^{13}C label was present in the starting material triketide-NAC ester as well as in the hydrolyzed triketide acid.

Studies on cell free production of the pigments 18 and 19 by *A. nidulans lovB*

The LovB CFE (15 mL) and LovB boiled control (10 mL) were prepared as described above (Method A). To the enzyme solutions were added FAD (5 mg), NADPH (5 mg), SAM (5 mg), Ac-CoA (5 mg), malonyl-CoA (0.5 mg) and 2-^{14}C malonyl-CoA (0.56 μCi, 1,250,000 DPM). The mixtures were placed on an orbital shaker (50 rpm) at rt for 5.5 h before adding half of a solution of **18** (5.5 mg) and **19** (3.6 mg) in 1 mL EtOAc to each reaction. The mixtures were acidified with 6 M HCl to pH 2 and extracted with EtOAc (3 X 30 mL). The organic fractions were dried ($MgSO_4$), filtered and evaporated under reduced pressure to yield the crude extracts. The crude extracts were dissolved in 10-15 mL EtOAc and a spatula tip of 10% Pd/C was added followed by placing the mixture under an atmospheric pressure of H_2 for 12 h. The mixture was filtered though a pad of celite washing with EtOAc. The solvent was evaporated under reduced pressure to yield the crude reaction mixture. The mixtures were purified by HPLC as described for **18** and **19**. The peaks corresponding to **18** and **19** were not radioactive. The majority of radioactivity was contained in a peak corresponding to malonic acid.

Studies on cell and cell free production of the pigment 3 by *A. nidulans lovB*

The LovB mycelia were ground with a mortar and pestle in extraction buffer and diluted with 25 mL extraction buffer. The mixture was split into 3 aliquots. One sample was boiled for 20 min, the second sample was centrifuged for 30 min at 7500 rpm at 4 °C and decanted to yield a CFE. Half of the CFE was ultrafiltered (YM100, 100,000 MWCO). The fourth sample was that of the ground mycelia, cells included. These 4 fraction were all submitted to the same conditions as follows. To the enzyme solutions were added (FAD (5 mg), NADPH (5 mg), and SAM (5 mg) dissolved in 1 mL Milli-Q H_2O) and (Ac-CoA (5 mg), malonyl-CoA (1 mg) and 2-^{14}C malonyl-CoA (1.16 μCi, 2,600,000 DPM) dissolved in 0.5 mL Milli-Q H_2O). The mixtures were placed on an orbital shaker (50 rpm) at rt for 20 h. A solution of **3** (1.2 mg) in 2 mL MeOH was added to the mixtures and the mixtures acidified with 4 M HCl to pH 2. The mixtures were extracted with EtOAc (3 X 30 mL). The organic fractions were dried (Na_2SO_4), filtered and evaporated under reduced pressure to yield the crude extracts. The mixtures were dissolved in MeOH and purified by HPLC as described for **3**. The peak corresponding to **3** was not radioactive in all cases. The majority of radioactivity was contained in a peak corresponding to malonic acid.

Studies on cell free production of the pigment 3 by *A. nidulans lovB* grown from original spores (20 h)

The LovB CFE (15 mL) and the LovB boiled control (10 mL) were prepared as described above (Method A). To the enzyme solutions were added (FAD (5 mg), NADPH (5 mg), and SAM (6 mg) dissolved in 1 mL Milli-Q H_2O) and (Ac-CoA (5 mg), malonyl-CoA (2 mg) and 2-^{14}C malonyl-CoA (5 μCi, 17,000,000 DPM) dissolved in 0.5 mL Milli-Q H_2O). The mixture was placed on an orbital shaker (50 rpm) at rt for 20 h. A solution of **3** (2.1 mg in 0.5 mL MeOH to the blank and 2.4 mg in 0.5 mL MeOH to the reaction) was added to the mixtures and the mixtures acidified with 4 M HCl to pH 2. The mixtures were extracted with EtOAc (3 X 30 mL). The organic fractions were dried (Na_2SO_4), filtered and evaporated under reduced pressure to yield the crude extracts. The mixtures were dissolved in MeOH and purified by HPLC as described for **3**. The peak corresponding to **3** was not radioactive in both cases. The majority of radioactivity was contained in peaks corresponding to malonic acid and malonyl-CoA.

Studies on cell free production of the pigment 3 by *A. nidulans lovB* grown from original spores (9 h)

The LovB CFE (15 mL) and the LovB boiled control (10 mL) were prepared as described above (Method A). To the enzyme solutions were added (FAD (5 mg), NADPH (5 mg), and SAM (6 mg) dissolved in 1 mL Milli-Q H_2O) and (Ac-CoA (5 mg), malonyl-CoA (2 mg) and 2-^{14}C malonyl-CoA (5 μCi, 17,000,000 DPM) dissolved in 0.5 mL Milli-Q H_2O). The mixture was placed on an orbital shaker (50 rpm) at rt for 9 h. A solution of **3** (1.6 mg in 0.5 mL MeOH to the blank and 3.3 mg in 0.5 mL MeOH to the reaction) was added to the mixtures and the mixtures acidified with 4 M HCl to pH 2. The mixtures were extracted with EtOAc (3 X 30 mL). The organic fractions were dried (Na_2SO_4), filtered and evaporated under reduced pressure to yield the crude extracts. The mixtures were dissolved in MeOH (1 mL) and a portion (100 μL) purified by HPLC as described for **3**. The peak corresponding to **3** was slightly radioactive (404 dpm). To verify the radioactivity, another 100 μL of crude was injected and the peak corresponding to **3** was collected and the solvent evaporated under reduced pressure. The residue was dissolved in MeOH and all of it was injected onto the HPLC. The peak corresponding to **3** counted only 32 dpm indicating the radioactivity was not significant, but was just radioactive bleed from the earlier peaks corresponding to malonic acid and malonyl-CoA.

Studies on isolation of the pyrones from fermentation of *A. nidulans lovB* as their methyl ethers 67 and 37

2 L of *A. nidulans lovB* were grown and expressed. Once the fermentation was complete the culture medium was extracted with EtOAC (thrice) and CH_2Cl_2 (once). The combined organic layers were dried (Na_2SO_4), filtered and the solvent evaporated under reduced pressure to give 110 mg of crude extract. K_2CO_3 (682 mg, 5.0 mmol) was added to the crude extract (110 mg, ~0.50 mmol) in dry acetone (4 mL) and the reaction mixture stirred for 20 min. MeI (314 μL, 5.0 mmol) was added and the reaction mixture stirred for 14 h. The mixture was filtered and the filtrate evaporated under reduced pressure. The crude material was purified by flash chromatography (SiO_2, 2:1/EtOAc:pentane). A fraction possibly containing the methylated pyrones was subjected to HPLC (same method as for **37**) however no peaks corresponding to (**67** or **37**) were identified.

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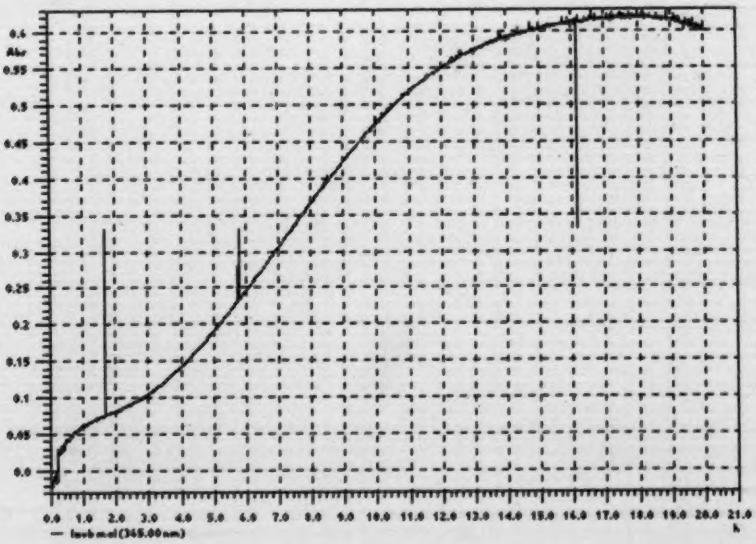
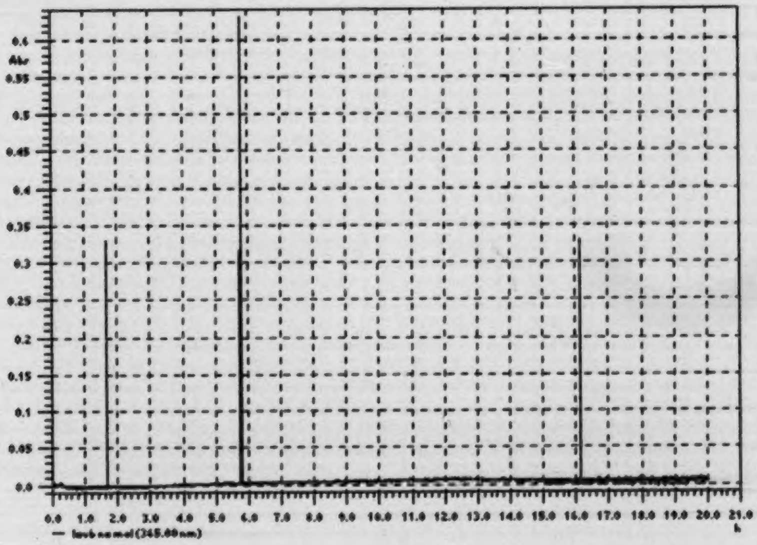
Appendix A

UV trace of LovB cell free extract enzyme activity assay with malonyl-CoA, Ac-CoA, NADPH, FAD and SAM



UV trace of LovB cell free extract enzyme activity assay control with NADPH, FAD and SAM, but missing malonyl-CoA and Ac-CoA,



Appendix B

Calculations and data for determining the secondary deuterium kinetic isotope effects for the ring closure of **74** and **78**.

1	Peak Area 236	Peak Area 237	Peak Area 237 - 16.7%	area 236/237
endo	17885092	23727937	19765371.52	0.904870014
exo	58555055	75128183	62581776.44	0.935656645
enz	770543	1012802	843664.07	0.913329169

2	Peak Area 236	Peak Area 237	Peak Area 237 - 16.7%	area 236/237
endo	23313933	31136837	25936985.22	0.898868269
exo	79525692	102669826	85523965.06	0.929864418
enz	(447979)	1149758	957748.414	0.467741834

3	Peak Area 236	Peak Area 237	Peak Area 237 - 16.7%	area 236/237
endo	16724435	21745144	18113704.95	0.923302828
exo	59842083	76027316	63330754.23	0.944913474
enz	679240	850127	708155.791	0.959167472

1	P (236/237) SM (H/D)
endo	0.901936399
exo	0.93262322
enz	0.910368129

SM	SM (mmol)	SM (H/D)
H (74)	0.0162861	1.003252573
D (78)	0.0162333	

2	P (236/237) SM (H/D)
endo	0.895954112
exo	0.926849771
enz	

3	P (236/237) SM (H/D)
endo	0.920309454
exo	0.941850038
enz	0.956057824

	Average k_H/k_D	
endo	**0.906**	
exo	**0.934**	**excluding run 2**
enz	0.622	**0.933**